REMARKS

Formal Matters

Claims 1-10 and 19-42 are pending after entry of the amendments set forth herein.

Claims 1-10 and 19-42 were examined. Claims 1-10 and 19-31 were allowed, and Claims 32-42 were rejected.

Claim 38 has been amended. Support for the amendment can be found in the claims as originally filed and throughout the specification at, for example, page 19, paragraph 82, page 21.

Applicants respectfully request reconsideration of the application in view of the amendments and remarks made herein.

No new matter has been added.

Allowable Subject Matter

Applicants wish to extend their gratitude to the Examiner for indicating that claims 1-10 and 19-31 are directed to allowable subject matter.

Rejections Under §112, First Paragraph

Claims 32-37

Claims 32-37 have been rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to comply with the written description requirement. This rejection is respectfully traversed as applied and as it may be applied to the pending claims.

In particular, the Office Action states the claims contain subject matter which was not described in such way as to reasonable convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action further states the following:

> However, there does not appear to be support in the instant application for the limitation, "contacting a candidate agent with a eukaryotic cell *in vitro*, wherein the contacting is performed in the presence of *an* agent that blocks nuclear export" (examiner's emphasis added). The passages cited by the response as provided support for the claimed methods (i.e. page 34, paragraph 132 through page 38, paragraph 141) only appear to provide support for a single

compound, HDAC3, that acts to block nuclear export in the working examples.

(Office Action, page 3). However, Applicants respectfully disagree.

Applicants first note that the compound HDAC3 is not an agent that blocks nuclear export. As provided in the specification, HDAC3 selectively deacetylates the RelA subunit of NF-κB and ultimately promotes nuclear export of the NF-κB complex (page 9, paragraph [0047]).

With respect to compounds that block nuclear export, Applicants note that the specification provides at least two examples of agents, leptomycin B (LMB) and trichostatin A (TSA), which block nuclear export (see specification on page 34, paragraph 132 through page 38, paragraph 141. Moreover, Figure 3I also shows that in the presence of leptomycin B or trichostatin A, nuclear export of GFP-RelA is blocked.

Furthermore, research articles published prior to the filing of the present application further support Applicants argument that other agents that block nuclear export were well known in the art at the time the present application was filed. For example, Finley et al., (JBC 104:189-200 (1987) (Exhibit A)) discloses that the lectins (e.g., wheat germ agglutinin) inhibit nuclear export transport (see abstract). In addition, Pasquinelli et al., (PNAS, 94:14394-14399 (1997) (Exhibit B)) discloses nuclear export signal conjugates which inhibit nuclear export in vertebrate cells. Specifically, Pasquinelli et al. discloses that when a nuclear export signal from either protein kinase A inhibitor protein or the HIV-1 Rev protein are used, nuclear export is blocked (see abstract, Figure 1, and page 14399, first column, third paragraph). Therefore, one skilled in the relevant art would have understood the inventors to be in possession of the claimed invention at the time the application was filed.

Accordingly, Applicants respectfully request that this rejection be withdrawn.

Claims 38-42

Claims 38-42 have been rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to comply with the written description requirement. This rejection is respectfully traversed as applied and as it may be applied to the pending claims.

In particular, the Office Action states the following:

Claim 38 recites the phrase "contacting the cell with an antibody that specifically binds acetylated RelA, wherein the contacting is under conditions sufficient for binding of the antibody to detect a level of acetylated RelA". The term "specifically binds acetylated RelA" are not clearly defined in the instant specification. As the claim is currently written, the term appears to be indefinite in that there is no explicit definition provided by the instant specification of what constitutes an antibody that "specifically binds" to acetylated RelA.

(Office Action, pages 3 and 4). However, Applicants respectfully disagree.

Applicants note that the specification provides ample support for claim amendments. In particular, the specification at, for example, on page 21, paragraph 88, provides that "it is possible to use an antibody to detect the presence of acetylated RelA". Moreover, the specification provides examples of such antibodies on page 21, paragraph 88, and page 31, paragraph 122, through page 32, paragraph 124.

However, in the spirit of expediting prosecution and without conceding to the correctness of the rejection, Claim 38 has been amended to remove the objectionable language and recite "contacting the cell with an anti-acetylated lysine antibody that binds acetylated RelA to detect a level of acetylated RelA". Support for the amendment can be found in the claims as originally filed and throughout the specification at, for example page 19, paragraph [0082], which states the following:

> Acetylation levels of RelA may be detected by using an anti-acetylated lysine antibody. The effect of a candidate agent on the acetylation level of RelA can by detected by using this antibody.

Accordingly, no new matter is introduced by this amendment.

As such, in the view of the remarks made herein and the amendments to the claims, Applicants respectfully request that this rejection be withdrawn.

Rejections Under §112, Second Paragraph

Claims 38-42 were rejected as being vague and indefinite for recitation of "contacting the cell with an antibody that specifically binds RelA wherein the contacting is under conditions sufficient for binding of the antibody to detect a level of acetylated RelA." This rejection is respectfully traversed as applied and as it may be applied to the pending claims.

Applicants respectfully submit that one of ordinary skill in the art would understand that, in the context of antibodies, an antibody that "specifically binds" a given antigen (here, acetylated RelA) to mean an antibody that has a greater affinity or avidity for the recited antigen than for another antigen. Indeed, the Merriam Webster Online Dictionary defines "specific" as follows (see in particular definition 2b):

> **1 a :** constituting or falling into a specifiable category **b :** sharing or being those properties of something that allow it to be referred to a particular category
> **2 a :** restricted to a particular individual, situation, relation, or effect <a disease *specific* to horses> **b :** exerting a distinctive influence (as on a body part or a disease) <*specific* antibodies>
> **3 :** free from ambiguity **:** **ACCURATE** <a *specific* statement of faith>
> **4 :** of, relating to, or constituting a species and especially a biologic species
> **5 a :** being any of various arbitrary physical constants and especially one relating a quantitative attribute to unit mass, volume, or area **b :** imposed at a fixed rate per unit (as of weight or count) <*specific* import duties> -- compare AD VALOREM

(italics in original, underlining added for emphasis)

However, without conceding to the grounds of this rejection, claim 38 is amended, rendering this rejection moot.

Withdrawal of this rejection is respectfully requested.

Conclusion

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number UCAL-234.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Nov. 23, 2004

By: _____
Edward J. Baba
Registration No. 52,581

Enclosures:

Exhibit A:	Finley et al., JBC 104:189-200 (1987)
Exhibit B:	Pasquinelli et al., PNAS, 94:14394-14399 (1997)

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Inhibition of In Vitro Nuclear Transport by a Lectin That Binds to Nuclear Pores

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Abstract. Selective transport of proteins is a major mechanism by which biochemical differences are maintained between the cytoplasm and nucleus. To begin to investigate the molecular mechanism of nuclear transport, we used an in vitro transport system composed of a *Xenopus* egg extract, rat liver nuclei, and a fluorescently labeled nuclear protein, nucleoplasmin. With this system, we screened for inhibitors of transport. We found that the lectin, wheat germ agglutinin (WGA), completely inhibits the nuclear transport of fluorescently labeled nucleoplasmin. No other lectin tested affected nuclear transport. The inhibition by WGA was not seen when *N*-acetylglucosamine was present and was reversible by subsequent addition of sugar. When rat liver nuclei that had been incubated with ferritin-labeled WGA were examined by electron microscopy, multiple molecules of WGA were found bound to the cytoplasmic face of each nuclear pore. Gel electrophoresis and nitrocellulose transfer identified one major and several minor nuclear protein bands as binding ^{125}I-labeled WGA. The most abundant protein of these, a 63–65-kD glycoprotein, is a candidate for the inhibitory site of action of WGA on nuclear protein transport. WGA is the first identified inhibitor of nuclear protein transport and interacts directly with the nuclear pore.

THE transport of macromolecules between the nucleus and cytoplasm appears to occur through the nuclear pores (Stevens and Swift, 1966; Feldherr et al., 1984). Structural studies based on electron microscopic observations indicate that the pore is composed of two prominent rings, one of which is located on the cytoplasmic surface of the nuclear envelope and the other on the nucleoplasmic surface. These rings define the periphery of the pore, each ring being composed of eight globular subunits (Maul, 1977; Franke et al., 1981; Unwin and Milligan, 1982). Located in the center of the pore is a central granule that appears to be connected to the ring subunits by spokes (Unwin and Milligan, 1982). At the molecular level, Gerace et al. (1982) have identified a concanavalin A (Con A)–binding glycoprotein of 180 kD molecular mass located at or near the nuclear pore. Fisher and colleagues have proposed that a 190-kD Mg^{++}-ATPase may also be a functional pore component (Berrios et al., 1983; Berrios and Fisher, 1986; Fisher, 1987). Most recently, Davis and Blobel (1986) identified a 62-kD protein that is a constituent of the nuclear pore. Although much is known about the structural morphology of the nuclear pore, relatively little is known about the pore proteins that play an active role in the transport of molecules through the pore.

The effective diameter of the nuclear pore has been measured as ~90Å (Paine et al., 1975). Macromolecules of small diameter are able to enter the nucleus by passive diffusion, whereas macromolecules of large diameter cannot (Bonner, 1975; Feldherr and Ogburn, 1980; Einck and Bustin, 1984; for reviews see Paine and Horowitz, 1980; De Robertis, 1983; Dingwall, 1985). Nuclear proteins of large size contain one or more signal sequences that direct them to the nucleus, possibly by an active transport mechanism (Dingwall et al., 1982; Hall et al., 1984; Kalderon et al., 1984*a*, *b*). During transport, the signal sequence of the nuclear protein is thought to interact either with the nuclear pore itself or possibly with a carrier protein, which then ferries the protein to or through the nuclear pore.

The *Xenopus laevis* nuclear protein, nucleoplasmin, has been the transport substrate of choice in a number of studies. Nucleoplasmin is an abundant oocyte nuclear protein of ~150 kD molecular mass and is composed of five identical subunits. Because it is easy to isolate, is stable, and is transported efficiently into the nucleus (Mills et al., 1980; Krohne and Franke, 1980; Dingwall et al., 1982), it is an ideal substrate for nuclear transport studies. Radiolabeled nucleoplasmin has been shown to accumulate to high levels within the oocyte nucleus when injected into oocyte cytoplasm and such accumulation requires a signal domain (Dingwall et al., 1982). Feldherr et al. (1984) have shown that nucleoplasmin–gold complexes enter the nucleus through the nuclear pore. Recently, Newmeyer et al. (1986*b*) found, using an autoradiographic assay, that radiolabeled nucleoplasmin was transported into synthetic nuclei and that such transport required ATP. We have subsequently developed an in vitro fluorescence assay that allows us to follow the transport of rhodamine isothiocyanate (RITC)-labeled nucleoplasmin into a nucleus as it occurs (Newmeyer et al., 1986*a*).

We have used this rapid in vitro assay of nuclear protein

transport to screen for inhibitors of transport. The assay, which uses an extract of *Xenopus* eggs, added rat liver nuclei, and RITC-labeled nucleoplasmin, allows the direct microscopic observation of transport within 30 min of the addition of nucleoplasmin. The in vitro assay faithfully mimics in vivo nuclear transport. Using this assay, we have identified an inhibitor of nuclear protein transport, the lectin wheat germ agglutinin (WGA).[1] Electron microscopy using ferritin-labeled WGA supports a direct interaction of this inhibitor with the nuclear pore. Our results indicate that the pore contains a novel glycoprotein that plays an essential role in the mechanism of nuclear protein transport. Further experiments point to a nuclear glycoprotein of 63–65 kD as a possible target of action for WGA in inhibition of nuclear transport.

Materials and Methods

Materials

Tetramethyl rhodamine-labeled nucleoplasmin was isolated essentially by the method of Dingwall et al. (1982), and was RITC-labeled as described by Newmeyer et al. (1986*a*). Fluorescein isothiocyanate (FITC)-labeled lectins were purchased from Polysciences, Inc., Warrington, PA. *N,N',N''*-triacetyl chitotriose was obtained from Sigma Chemical Co., St. Louis, MO. Unlabeled WGA and *N*-acetyl-D-glucosamine were obtained from Calbiochem Behring Corp., La Jolla, CA. An autoimmune antilamin antiserum, which reacts with the rat nuclear lamins A and C, was a gift from Frank McKeon (Harvard University Medical School, Boston, MA). Iodobeads were purchased from Pierce Chemical Co., Rockford, Illinois.

Preparation of Nuclei, Nuclear Fractions, and Egg Extracts

Rat liver nuclei were prepared essentially by the method of Blobel and Potter (1966) with slight modifications and the addition of 0.5 mM spermidine. Rat liver nuclei (1–5 × 10^5/μl) were stored frozen at −70°C in the same buffer plus 250 mM sucrose.

Rat liver nuclear envelopes (ghosts) were isolated as described by Dwyer and Blobel (1976). Demembranated sperm nuclei were prepared by the method of Lohka and Masui (1983) and stored frozen at −70°C at a concentration of 1–4 × 10^5/μl. *Xenopus* embryonic nuclei, isolated from 9-h-old embryos, were prepared by gently Dounce-homogenizing the embryos and centrifuging the embryo extract in a clinical centrifuge to remove yolk granules. The majority of embryonic nuclei were not removed by this centrifugation and could be assayed for transport by the addition of tetramethylrhodamine isothiocyanate (TRITC)-labeled nucleoplasmin and an ATP-regenerating system. Nuclei reconstituted from bacteriophage DNA were prepared as described in Newmeyer et al. (1986*a*) and in Newport, 1987.

Xenopus egg extracts were prepared essentially by the method summarized in Newport and Forbes (1985). *Xenopus* eggs were dejellied with a 5-min incubation in 2% cysteine, pH 8, activated with the calcium ionophore A23187, and packed and lysed by centrifugation in buffer containing 250 mM sucrose, 50 mM KCl, 1 mM DTT, 2.5 mM MgCl, 0.02 mg/ml cycloheximide, and 0.005 mg/ml cytochalasin B. The cleared extract was recentrifuged and used for the transport assay either immediately or within several hours if stored on ice.

Nucleoplasmin Transport Assay

Transport was assayed as described (Newmeyer et al., 1986*a*). Briefly, nuclei (0.5–3 × 10^5 in 1 μl) were added to 20 μl of egg extract supplemented with 1–2.75 mM ATP, 9 mM creatine phosphate, and 100 U/μl of creatine kinase. For assays using rat liver nuclei, the nuclei were incubated in the extract for 30 min to allow equilibration with the extract and/or healing of any small perforations. At this time 1 μl of TRITC-labeled nucleoplasmin (final concentration, ~15 ng/μl) was added. Transport of nucleoplasmin was assayed microscopically by taking aliquots at various times after nucleoplasmin addition. For this, 4–5 μl of the transport reaction mixture was placed on a slide and mixed with 0.5 μl 37% formaldehyde and 0.5 μl of 10 μg/ml bisbenzimide DNA dye (Hoechst 33258) before the coverslip was added. For experiments with demembranated sperm nuclei, the sperm nuclei were added to the extract and allowed to reacquire a nuclear envelope, decondense their DNA, and swell to nuclei several times the size of rat liver nuclei (~30–60 min) before the addition of TRITC-labeled nucleoplasmin.

Effects of Lectins on Nuclear Transport

To assay for binding of FITC-labeled lectins to nuclei in egg extract, one of two procedures that gave equivalent results was followed. In one, the labeled lectin was added to the extract to a concentration of 0.1 mg/ml and the nuclei observed microscopically 30 min later. In the second, the FITC-labeled lectin was added (0.5 μl of a 1 mg/ml stock solution) to a slide with a 5-μl sample of extract containing nuclei and the mixture immediately examined.

To assay the effect of lectin addition on transport, rat liver nuclei were added to 20 μl of egg extract (final concentration of 1–5 × 10^5 nuclei/20 μl) and allowed to incubate for 20 min. At this time, FITC lectin (1 mg/ml in PBS) was added to the extract (final concentration of 0.1 mg/ml) and incubated for 5 min at ambient temperature. An equivalent volume of PBS was added to control incubations instead of lectin. After the 5-min preincubation with lectin, 1/20th volume of nucleoplasmin was added to a final concentration of 15 μg/ml. Aliquots were assayed microscopically for accumulation or lack thereof 20 min later. In some incubations, *N*-acetyl-D-glucosamine was added to a final concentration of 0.5 M before the addition of WGA, or *N,N',N''*-triacetyl chitotriose was added to a concentration of 1 mM.

To determine whether the inhibition observed with WGA resulted from an interaction of the lectin with the nuclei or, instead, with the extract, 10 μl of rat liver nuclei were added to 100 μl of egg extract. WGA (50 μl) was added to one-half of this mixture (final concentration 0.1 mg/ml), and 50 μl of PBS was added to the other half. Each was allowed to incubate for 5 min at ambient temperature before dilution fivefold with buffer (80 mM KCl, 5 mM EDTA, 15 mM Pipes, pH 7.0, 200 mM sucrose, 7 mM $MgCl_2$). The diluted nuclei were layered on a 15/40% Percoll step gradient, which was then centrifuged at 1000 *g* for 10 min. The nuclei, which band at the interface between the two Percoll phases, were removed, diluted fivefold (final volume 300–500 μl) with the above buffer, and pelleted for 30 s in a microfuge. The nuclear pellet (1–2 μl) was resuspended in 20 μl of egg extract and incubated for 20 min before addition of 1/20th volume of TRITC-labeled nucleoplasmin. The percent of nuclei accumulating nucleoplasmin in the nuclei preincubated with WGA and the control nuclei was assayed microscopically in the usual manner.

Fluorescence Microscopy

Samples were observed using a Zeiss Photomicroscope II fitted for fluorescence visualization of FITC, RITC, and the DNA dye, bisbenzimide. The level of TRITC-nucleoplasmin accumulation in a single accumulating nucleus relative to external concentration could be quantitated by densitometric scanning of photographic negatives.

Gel Electrophoresis, Immunoblotting, and Radiolabeled Lectin Blotting

The proteins present in nuclear or extract samples were prepared for gel electrophoresis by solubilization in 10% glycerol, 4% SDS, 0.125 M Tris-HCl, pH 6.8, 0.5% bromophenol blue, and 0.05% 2-mercaptoethanol, and by boiling for 3 min. The solubilized samples were loaded onto 10% polyacrylamide SDS gels prepared by the method of Laemmli (1970) and electrophoresed at 200 V for 4 h. The proteins were electrophoretically transferred onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) using a Bio-Rad transblot apparatus (100 V for 4 h to overnight) in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS, and 20% methanol.

Protein blots were probed for the presence of WGA-binding glycoproteins by incubation with ^{125}I-labeled WGA using the method of Bartles and Hubbard (1984), which involves incubation at 4°C in 15 ml of PBS containing 2% polyvinylpyrrolidone and 0.5–4 × 10^6 cpm (per 15 ml) of iodinated WGA. The method of incubation was slightly modified in that the polyvinyl-

1. *Abbreviations used in this paper*: GlcNAc, *N*-acetylglucosamine; WGA, wheat germ agglutinin.

pyrrolidone used was either 360 or 40 kD in size and periodate-aniline-cyanoborohydride treatment was omitted. The blots were exposed for autoradiography with Kodak X-OMAT AR5 film.

To prepare iodinated WGA, Iodobeads were preincubated with 0.5 mCi of ^{125}I-iodine (Amersham Corp., Arlington Heights, IL) for 5 min at ambient temperature in 0.1 M potassium phosphate, pH 7.5, 0.2 M *N*-acetyl-D-glucosamine. WGA in 0.1 M potassium phosphate, pH 7.5, 0.2 M *N*-acetyl-D-glucosamine was added to a concentration of 2 mg/ml and incubated for 15 min at ambient temperature. The supernatant was removed from the Iodobead to stop the reaction, and passed twice over a Sephadex G25 Fine column (Pharmacia Fine Chemicals, Piscataway, NJ), which had been equilibrated in 0.1 M potassium phosphate, pH 7.5, to remove unbound ^{125}I-iodine.

The position of the nuclear lamin proteins A and C on the protein blots was determined by incubating a blot identical to that probed with radioactive WGA with antilamin antiserum. For this, the blot was first incubated with 5% bovine serum albumin, 0.1% Tween 20 in PBS for 1 h at ambient temperature. The blot was then incubated for 2 h with 15 ml of a 1:15,000 dilution of antilamin antiserum (LSI; McKeon et al., 1983) in 5% bovine serum albumin, 0.1% Tween 20 in PBS. The blot was washed three times in the same buffer but with no added antiserum and twice with PBS alone. The washed blot was incubated for 2 h at ambient temperature with ^{125}I-labeled protein A (0.033 mg/ml) at a concentration of 2–4 × 10^6 cpm/15 ml. After this incubation, the blot was washed as after the incubation with antiserum and exposed for autoradiography.

Electron Microscopy

Rat liver nuclei were diluted in PBS to a concentration of 0.2–1 × 10^5/μl. In some samples, 1 mM *N,N',N"*-triacetyl chitotriose was added. Ferritin-labeled WGA (0.1 mg/ml; Polysciences, Inc.) was added and allowed to incubate for 20 min. At this time, samples were fixed for 30 min on ice in 2% glutaraldehyde and 2.5% formaldehyde in 0.2 M cacodylate buffer (pH 7.4). The samples were centrifuged briefly to form a loose pellet. The fixative was discarded and the pellets resuspended in a small volume of 2% agarose at 30°C, followed by chilling. Samples were postfixed with 2% OsO_4 in 0.2% cacodylate, washed, dehydrated through a graded series of ethanol, and embedded in Spurr's low viscosity resin (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope operated at 60 kV with a 50-μm objective aperture.

Results

WGA Binding to the Nuclear Periphery

One approach to identifying molecular components involved in nuclear transport would be to first identify specific inhibitors of transport and then to ask which nuclear proteins those inhibitors bind to. Several studies have pointed towards a possible involvement of one or more glycoproteins in nuclear transport. For example, Gerace et al. (1982) identified a 180-kD Con A-binding glycoprotein located at the nuclear pore. In a separate study, two lectins, Con A and wheat germ agglutinin, were reported to block the ATP-dependent release of ribonucleoprotein from isolated rat liver nuclei (Baglia and Maul, 1983), a biochemical assay thought to reflect the export of ribonucleoproteins from the nucleus (Agutter et al., 1979). In contrast, a study by Jiang and Schindler (1986), which measured the influx of a dextran molecule into rat liver nuclei, found no effect of WGA, but found that dextran influx was inhibited by Con A.

To identify possible inhibitors of nuclear protein transport, we used an assay developed to allow us to measure transport quickly in vitro (Newmeyer et al., 1986*a*). This assay uses, as a transport medium, an extract of activated *Xenopus laevis* eggs that contains all the components, with the exception of DNA, necessary to assemble nuclei in vitro (Forbes et al., 1983; Newport and Forbes, 1985; Newport et al., 1985; Newmeyer et al., 1986*b*; Newport, 1987). For the assay, nuclei are added to the egg extract and allowed to equilibrate for 30 min, at which time RITC-labeled nucleoplasmin is added. Aliquots, removed and examined under the fluorescence microscope, show nuclear accumulation of TRITC nucleoplasmin within minutes, with accumulation reaching a maximum at 30–45 min. When isolated rat liver nuclei are added to this extract, the majority maintain a functional nuclear envelope by three criteria: (*a*) large fluorescently labeled non-nuclear proteins (fluorescein-labeled immunoglobulin; phycoerythrin) are excluded from the nuclei, indicating that the nuclear envelopes are intact; (*b*) the nuclear envelope grows in size, gaining an adjacent extension of nuclear membrane that contains components derived from the egg extract; and (*c*) the nuclei transport and accumulate the fluorescently labeled nuclear protein, TRITC nucleoplasmin, up to 17-fold. (The number of accumulating nuclei ranges from 50 to 90% of the nuclei added, with freshly isolated nuclei showing a higher percentage of accumulating nuclei.) Nucleoplasmin accumulation in this assay is specific in that the signal domain of nucleoplasmin is required. Moreover, transport is temperature- and ATP-dependent. Finally, transport is observed using nuclei that possess their own nuclear envelopes when added to the extract (rat liver nuclei, *Xenopus* embryonic nuclei) or nuclei that acquire a nuclear envelope from the egg extract (demembranated *Xenopus* sperm nuclei) (Newmeyer et al., 1986*a*). This assay thus mimics transport as it occurs in vivo, and, because of its convenience, is appropriate for the quick direct identification of nuclear transport inhibitors.

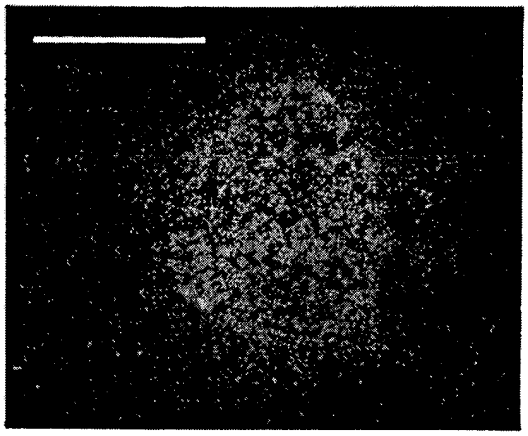

Figure 1. Punctate staining of a rat liver nuclear envelope by FITC-WGA. FITC-WGA was added to rat liver nuclei that had been incubated in egg extract. The view shown was photographed by focusing on the top surface of the nucleus. Bar, 10 μm.

Before testing a variety of lectins for possible inhibition of nucleoplasmin transport, we first asked whether specific lectins bound to rat liver nuclei under our conditions. Nuclei were added to an egg extract for 20 min. An aliquot of the mixture was placed on a slide, 0.5 μl of FITC-labeled lectin (0.1 mg/ml final concentration) added, and the aliquot examined by fluorescence microscopy. We found that WGA bound strongly to the nuclear envelopes of all rat liver nuclei. Interestingly, FITC-WGA stained the periphery of nuclei in a finely punctate manner (Fig. 1), even after fixation for 30 min with 2.5% glutaraldehyde (not shown). FITC-Con A stained the nuclear envelopes of damaged nuclei but not intact

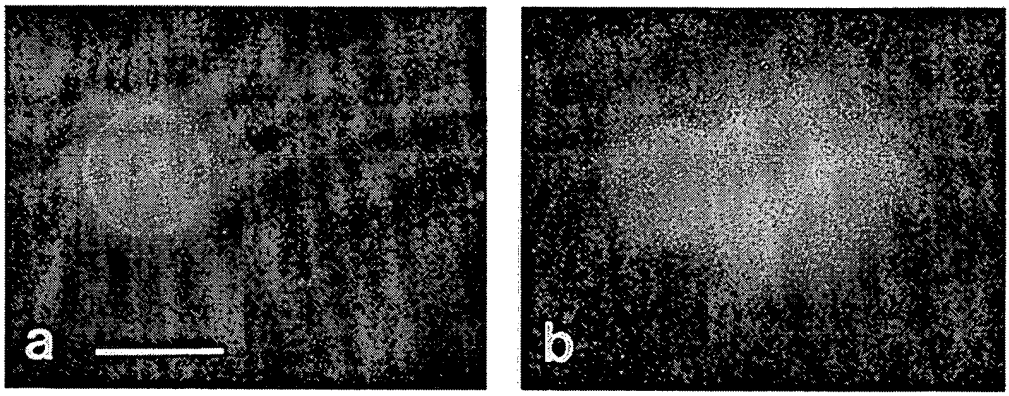

Figure 2. Differential staining of the original rat liver nuclear envelope and newly added nuclear envelope. Rat liver nuclei were added to an egg extract and allowed to equilibrate with the extract for 30 min. TRITC-labeled nucleoplasmin was then added. After 30 min, an aliquot was examined microscopically for accumulation and ability to bind FITC-labeled WGA by adding 0.5 μl FITC-WGA on the slide. The rat liver nucleus shown has undergone membrane growth; the small hemisphere derives from the original nucleus and contains the DNA, as determined by bisbenzimide staining (not shown). (*a*) FITC-WGA stains the original envelope brightly (the smaller hemisphere) and the newly added membrane weakly (larger hemisphere). (*b*) TRITC nucleoplasmin accumulation by the same rat liver nucleus as in *a*, shown to indicate the intactness of the nuclear envelope. Note: In this experiment, TRITC nucleoplasmin accumulation was allowed to occur for 30 min before the addition of WGA. Bar, 10 μm.

nuclei, as described previously (Newmeyer et al., 1986*a*). Unlike WGA, FITC-Con A stained the nuclear periphery in a continuous manner (see below). Other FITC-labeled lectins, *Bauhinia purpurea* agglutinin, *Dolichos biflorus* lectin, *Griffonia simplicifolia* lectins, *Maclura pomifera* lectin, *Arachis hypogaea* lectin, soy bean lectin, and *Ulex europaeus* lectin, gave no or only faint nuclear staining. Several of these lectins strongly stained non-nuclear membranous vesicles in the egg extract. Thus, of 10 lectins tested, only WGA and Con A were found to stain nuclei and did so at the nuclear periphery.

As stated, wheat germ agglutinin stained all rat liver nuclei. When rat liver nuclei are added to *Xenopus* egg extracts, they gain membranous extensions of their nuclear envelope. This envelope growth most often appears as an adjacent membrane bleb attached to the original nuclear envelope (Newmeyer et al., 1986*a*). We found that the newly added nuclear envelope that was acquired from the egg ex-

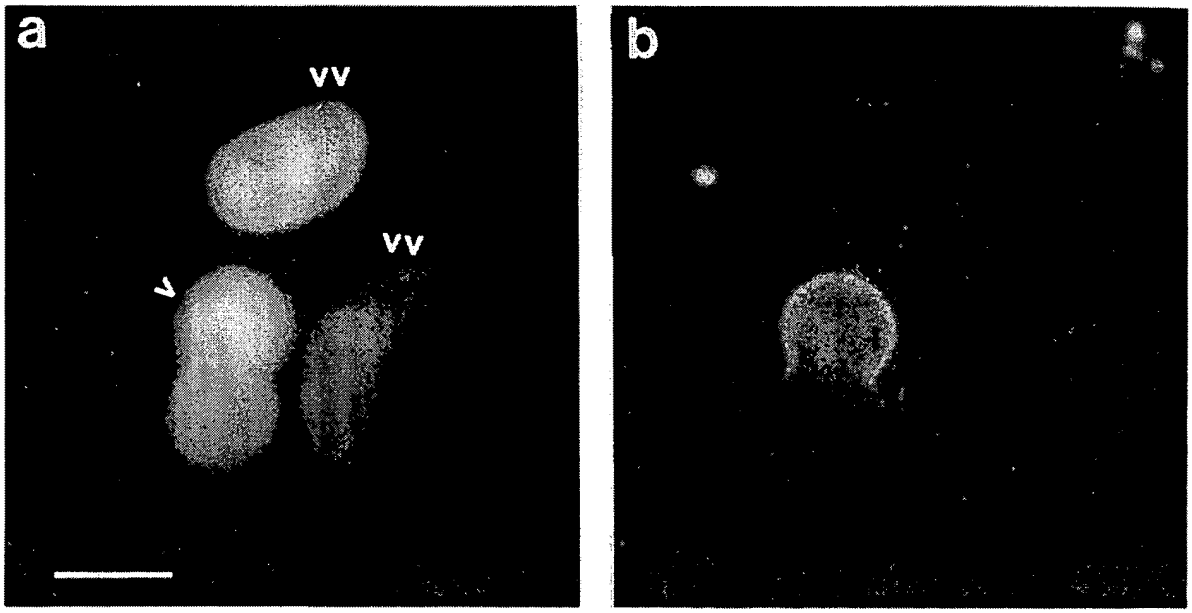

Figure 3. Differential staining of the nuclear envelopes of regrown sperm nuclei and rat liver nuclei. Demembranated *Xenopus* sperm nuclei were added to an egg extract. Within 30 min, the DNA decondensed and the nuclei acquired nuclear envelopes from components present in the extract. Rat liver nuclei were then added, followed by addition of TRITC-labeled nucleoplasmin. 30 min later, an aliquot was applied to a slide and FITC-WGA added. (*a*) TRITC nucleoplasmin accumulation is obvious in all three nuclei, indicating that intact nuclear envelopes are present. (*b*) FITC-WGA staining. Only the original rat nuclear envelope is stained brightly. The rat liver nucleus is designated by the single arrowhead and sperm nuclei by double arrowheads. Note: WGA was added 30 min after nucleoplasmin addition. Bar, 10 μm.

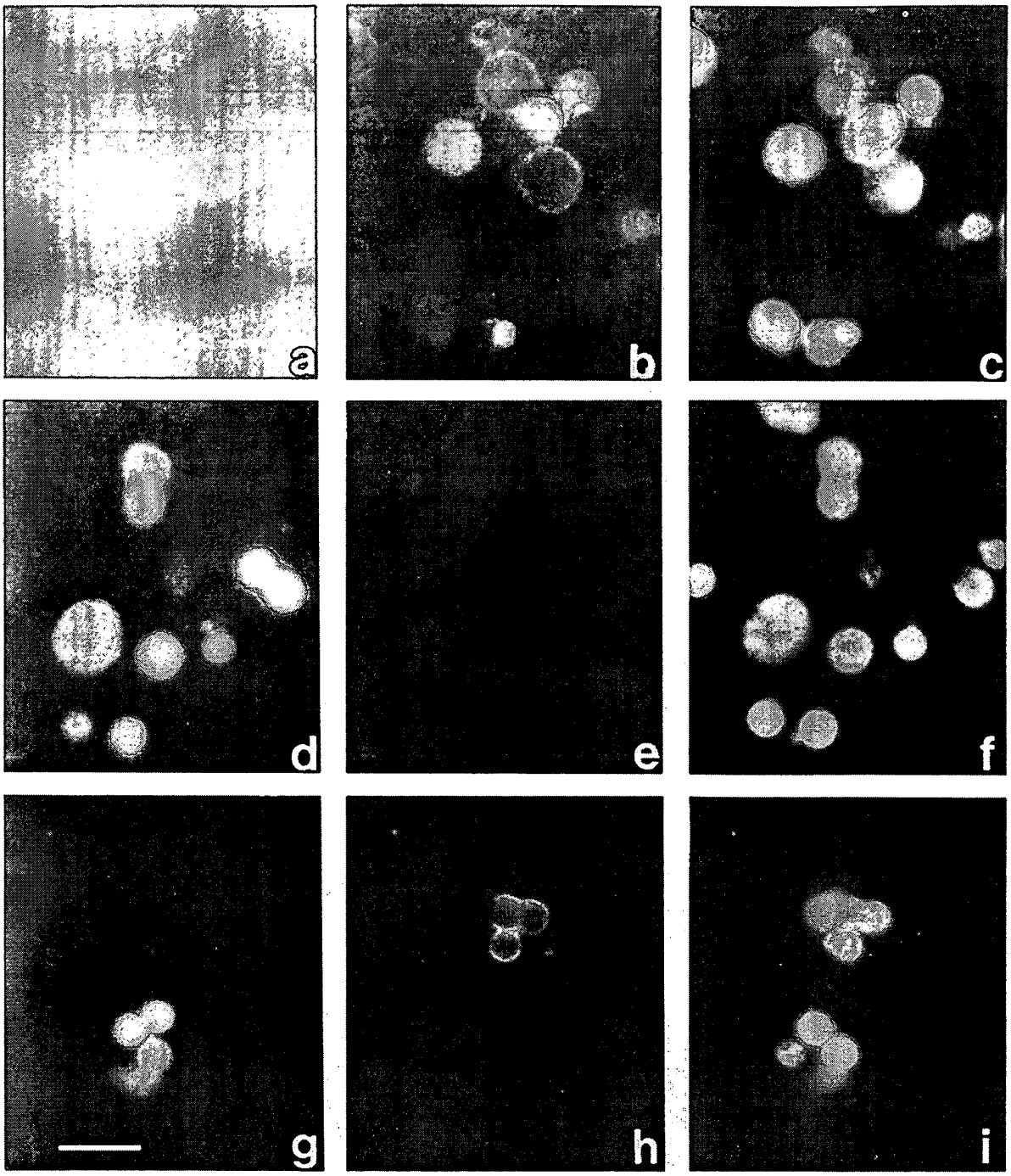

Figure 4. Nucleoplasmin transport is inhibited by the lectin WGA, but not by lectins Con A or *Arachis hypogaea*. Rat liver nuclei were added to egg extract and allowed to incubate for 30 min before the addition of WGA (*a–c*), *Arachis hypogaea* (*d–f*), or Con A (*g–i*) to a final concentration of 0.1 mg/ml. After 5 min, TRITC nucleoplasmin was added and aliquots observed by fluorescence microscopy 30 min later. (*a*, *d*, and *g*) TRITC nucleoplasmin fluorescence; (*b*, *e*, and *h*) FITC lectin fluorescence; (*c*, *f*, and *i*) DNA fluorescence as visualized with bisbenzimide. (The nuclei in *g–i* were chosen to include a number of broken nuclei to demonstrate FITC-Con A staining of broken nuclei. WGA addition results in the exclusion of TRITC nucleoplasmin (*a*) by intact nuclei, while PNA (*d*) and Con A (*g*) have no effect on TRITC nucleoplasmin accumulation. Bar, 20 μm.

tract stained more weakly than the original nuclear envelope, indicating that the added envelope is of a different protein composition (Fig. 2 *a*). To aid in visualizing the boundaries of the nuclear envelope in Fig. 2, TRITC nucleoplasmin (Fig. 2 *b*) was added to the extract containing nuclei 30 min before the addition of FITC-WGA. Efficient accumulation of nucleoplasmin can be seen to have taken place and fills both lobes of the enlarged rat liver nucleus. Thus, this nucleus is

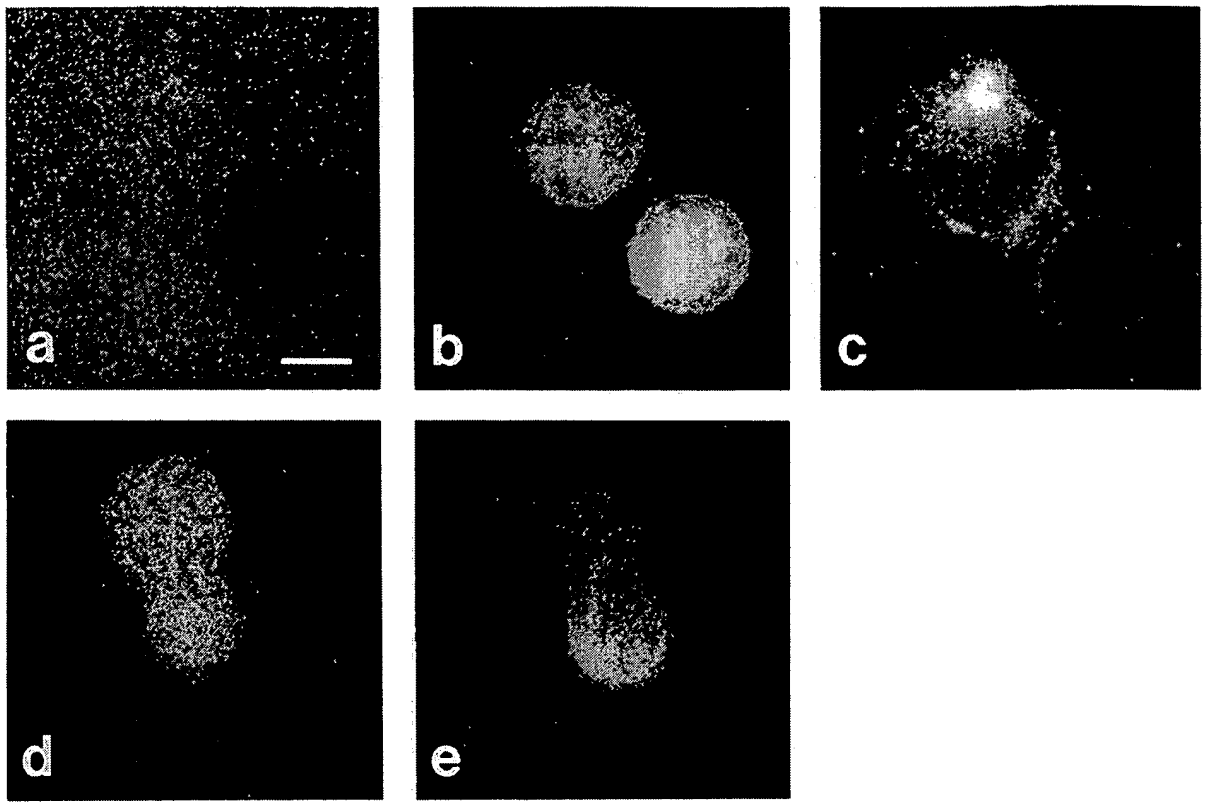

Figure 5. WGA inhibition of nucleoplasmin transport is reversed by incubation with a competing sugar. Rat liver nuclei were added to egg extract and allowed to incubate for 20 min before the addition of FITC-WGA to a final concentration of 0.1 mg/ml without (*a–c*) or with (*d* and *e*) co-addition of the competing sugar, *N*-acetyl-glucosamine (0.5 M). 5 min after lectin addition, TRITC nucleoplasmin was added and aliquots observed by fluorescence microscopy 30 min later. (*a* and *d*) TRITC nucleoplasmin fluorescence; (*b* and *e*) bisbenzimide DNA fluorescence; (*c*) FITC-WGA fluorescence. Bar, 10 μm.

intact and contains a hybrid nuclear envelope as revealed by subsequent FITC-WGA staining.

Consistent with the finding that FITC-WGA stains the newly added nuclear membrane more weakly, we found that regrown *Xenopus* sperm nuclei, which acquire a nuclear envelope derived exclusively from components in the egg extract (Lohka and Masui, 1983), also stained weakly with FITC-wheat germ agglutinin. To visualize directly the difference in staining intensity, demembranated *Xenopus* sperm nuclei were added to an egg extract and allowed to reacquire a nuclear envelope, decondense their DNA, and swell to sizes larger than rat liver nuclei. We previously showed that such nuclei are capable of TRITC–nucleoplasmin transport (Newmeyer et al., 1986*a*). Rat liver nuclei were then added to the extract containing regrown sperm nuclei. TRITC nucleoplasmin was added and accumulation allowed to take place. An aliquot containing both types of nuclei was then removed and tested for FITC-WGA binding. Fig. 3 shows a representative rat liver nucleus and two regrown sperm nuclei. All three have accumulated nucleoplasmin and thus contain intact nuclear envelopes (Fig. 3 *a*). Only the original nuclear envelope of the rat liver nucleus stains brightly with FITC-WGA (Fig. 3 *b*). The nuclear envelope contributed by the extract thus appears to contain less WGA-binding glycoproteins or to contain proteins altered in their glycosylation or accessibility to WGA. (Weak staining with FITC-WGA is not a characteristic of *Xenopus* nuclear envelopes in general, since the envelopes of *Xenopus* embryonic nuclei stained as brightly as those of rat liver nuclei; data not shown). We conclude that the amount, accessibility, or level of glycosylation of the glycoprotein(s) recognized by WGA in the nuclear envelope varies between nuclei of different types.

Inhibition of Nuclear Protein Transport by WGA

To test whether the nuclear binding of WGA affected transport of nucleoplasmin, lectin was added to the transport assay itself prior to the addition of TRITC-labeled nucleoplasmin. We found that the addition of wheat germ agglutinin, either FITC-labeled or unlabeled, completely blocked nucleoplasmin transport (Figs. 4, *a–c* and 5, *a–c*). Nuclei were often seen that clearly excluded TRITC nucleoplasmin (Figs. 4 *a* and 5 *a*), indicating that they were intact but incapable of transport. The inhibition of nucleoplasmin accumulation was complete at a concentration of 0.1 mg/ml WGA. Concentrations between 0.02 and 0.1 mg/ml gave partial inhibition, i.e., the amount of accumulation per nucleus was lower than in controls lacking WGA. When the concentration of WGA was reduced to 0.01 mg/ml WGA, no inhibition was seen (Table I).

When those lectins that failed to stain nuclei were tested, it was found that none blocked nucleoplasmin transport. The

high levels of nucleoplasmin accumulation observed with one such lectin, *Arachis hypogaea* lectin are shown in Fig. 4, *d–f*. FITC-Con A, as described previously (Newmeyer et al., 1986*a*) stained damaged rat liver nuclei at the nuclear periphery, but neither stained nor affected the transport of nucleoplasmin into intact nuclei (Fig. 4, *g–i*). Thus, only WGA was found to inhibit nuclear transport of fluorescently labeled nucleoplasmin.

Because FITC-WGA stained regrown *Xenopus* sperm nuclear envelopes more weakly than those of rat liver nuclei, it was possible that addition of WGA would not block nucleoplasmin accumulation in sperm nuclei. We found, however, that accumulation was as efficiently blocked in sperm nuclei as in rat liver nuclei (Table I). WGA also blocked nuclear accumulation of nucleoplasmin in *Xenopus* embryonic nuclei and nuclei reconstituted from bacteriophage DNA (Table I).

The inhibition of nucleoplasmin transport by WGA appears to be due to a specific interaction between WGA and a carbohydrate residue, since the inclusion of competing sugar (500 mM *N*-acetylglucosamine or 1 mM *N,N',N"*-triacetyl chitotriose) at the time of WGA addition resulted in completely normal nucleoplasmin transport (Fig. 5, *d–e*). Furthermore, it was possible to reverse WGA inhibition of nucleoplasmin transport by the addition of competing sugar 30 min later (Table I).

Wheat Germ Agglutinin Recognizes a Nuclear Pore Protein

To determine whether WGA was binding to a nuclear component or to an extract component, rat liver nuclei were briefly exposed to WGA, then washed twice with buffer before the transport assay. For this procedure, WGA was added to nuclei in extract and incubated for 5 min (0.1 mg/ml final concentration). Washes were then performed by dilution of the mixture and centrifugation on a 15/40% Percoll step gradient. The interface between the two Percoll phases was withdrawn, diluted, and centrifuged for 30 s in a microcentrifuge to wash the nuclei further. The pelleted nuclei were resuspended in fresh egg extract and incubated for 10 min. TRITC nucleoplasmin was then added and transport assayed 30 min later. Nuclei preincubated with WGA and washed in this manner exhibited no nucleoplasmin transport when added to fresh extract (Table II). Control nuclei, which were not preincubated with WGA, but which were subjected to the same washing procedure, were capable of efficient transport (Table II). This result indicates that WGA is binding to the nucleus itself and that this binding is sufficient to block the transport of subsequently added nucleoplasmin.

To determine the nuclear site of WGA binding, electron microscopy was performed. Rat liver nuclei in PBS were mixed with ferritin-labeled WGA for 20 min, then centrifuged to concentrate the nuclei. The pelleted nuclei were fixed, embedded, sectioned, and examined with the electron microscope. We found that ferritin WGA bound to the nuclei and did so almost exclusively on the cytoplasmic faces of the nuclear pores. Nearly all the nuclear pores (>95%) were decorated as in Fig. 6 *a*, *b*, *d*, and *e*, where numerous ferritin grains can be seen in each pore. Rare pores (<2%) also showed ferritin on the nucleoplasmic face of the pore, but ferritin binding to the nuclear membrane was not observed. The binding of ferritin WGA to the pores appears specific, since incubation of the nuclei with ferritin-labeled WGA and 1 mM *N,N',N"*-triacetyl chitotriose (a competing sugar) resulted in no ferritin WGA binding to the pores (<2%; Fig. 6, *c* and *f*). We conclude that WGA recognizes multiple copies of one or more glycoproteins present on the cytoplasmic face of the nuclear pore.

WGA-binding Nuclear Proteins

To identify the nuclear glycoprotein(s) to which WGA binds, proteins from rat liver nuclei, rat liver nuclear pore–lamina

Table I. Conditions Affecting Nucleoplasmin Accumulation

Rat liver nuclei	Accumulation
Control	+++
+ Con A (0.1 mg/ml)	+++
+ WGA (0.1 mg/ml)	–
+ WGA (0.05 mg/ml)	+
+ WGA (0.01 mg/ml)	+++
+ WGA + *N,N',N"*-triacetyl chitotriose	+++
+ WGA for 30 min, then *N,N',N"*-triacetyl chitotriose	+++
+ lectins BPA, DBA, MPA, GS I and II, PNA, SBA, or UEA I (0.1 mg/ml)	+++
Xenopus sperm nuclei	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–
Xenopus embryonic nuclei	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–
Nuclei reconstituted from bacteriophage DNA	
RITC nucleoplasmin	+++
RITC nucleoplasmin + WGA (0.1 mg/ml)	–

Transport assays were performed as described in Materials and Methods. Lectins were added, where noted, 5 min before nucleoplasmin addition. Nucleoplasmin accumulation was measured 30 min after addition of nucleoplasmin. Several hundred nuclei in multiple experiments were monitored for each of the conditions tested. The concentration of WGA was 0.1 mg/ml, except where noted. BPA, *Bauhinia purpurea* agglutinin; DBA, *Dolichos biflorus* lectin; MPA, *Maclura pomifera* lectin; GS I and II, *Griffonia simplicifolia* lectins; PNA, *Arachis hypogaea* lectin; SBA, soybean lectin; UEA I, *Ulex europaeus* lectin; +++, high levels of accumulation (up to 17-fold); +, accumulation that was visible but faint (approximately twofold); –, no accumulation seen in any of the nuclei examined.

Table II. Inhibition of Nuclear Transport by WGA

	WGA	Sugar	Accumulated nuclei	Total nuclei	Accumulated nuclei
			No.		%
Rat liver nuclei					
	–	–	41.0	87.0	47.0
	+	–	0.0	82.0	0.0
	+	+	37.0	84.0	44.0
Washed rat liver nuclei					
	–	–	23.0	62.0	37.0
	+	–	1.0	60.0	1.6

Transport assays were performed as described in Materials and Methods. Nuclei accumulating RITC-labeled nucleoplasmin were visualized by their rhodamine fluorescence. The total number of nuclei was determined by staining for DNA with the fluorescent DNA dye bisbenzimide. Observations were made 30 min after the addition of nucleoplasmin. When present, WGA and *N,N',N"*-triacetyl chitotriose were added 5 min before the addition of nucleoplasmin.

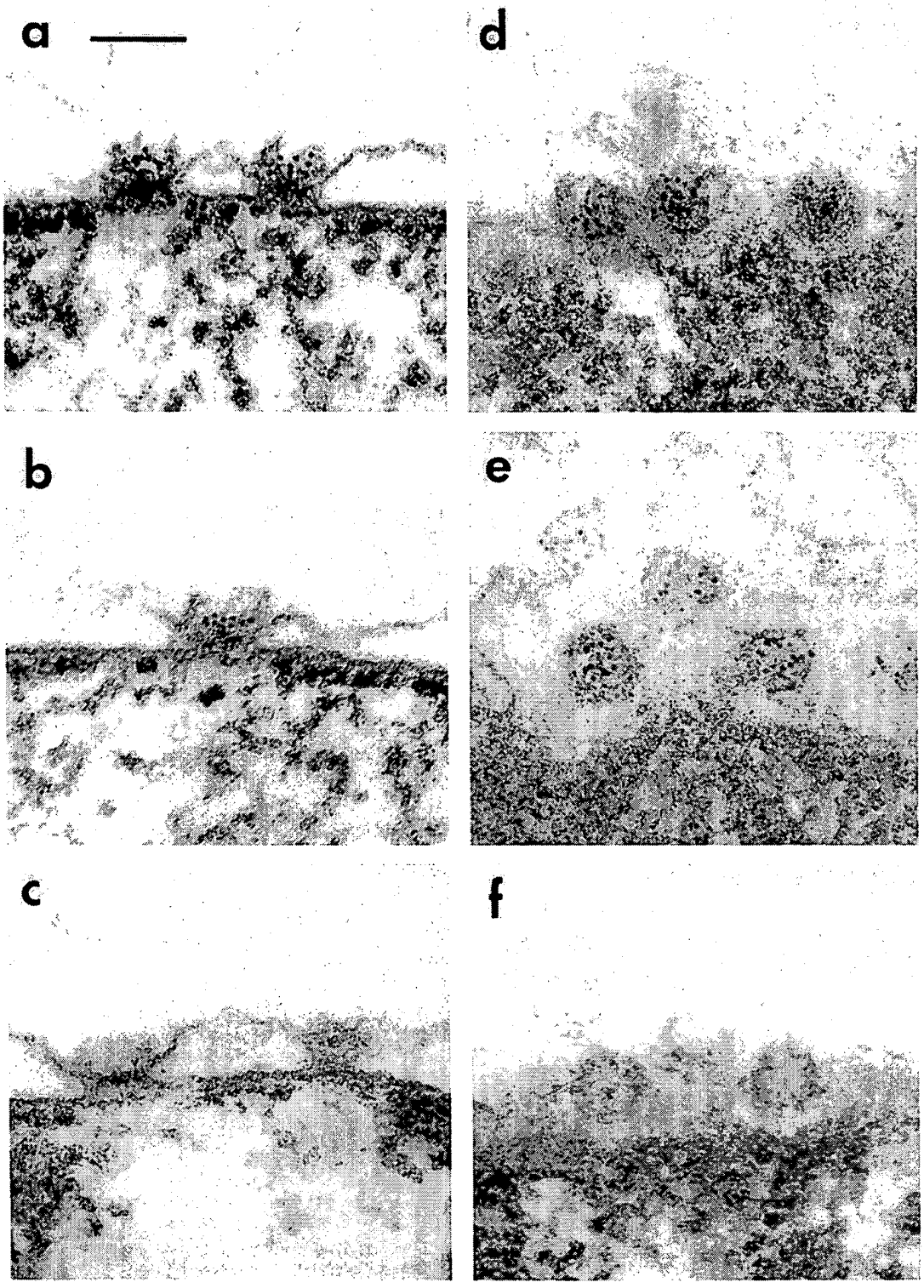

Figure 6. Ferritin-labeled WGA binds to the cytoplasmic side of the nuclear pore. Rat liver nuclei were incubated with ferritin-labeled WGA in PBS before preparation for electron microscopy. Control samples were incubated in PBS containing 1 mM *N,N',N"*-triacetyl chitotriose. *a*, *b*, *d*, and *e* show rat liver nuclear pores incubated with ferritin-labeled WGA, while panels *c* and *f* show nuclear pores of nuclei incubated with ferritin WGA and the competing sugar, chitotriose. (*a* and *b*) Cross sections of representative nuclear pores containing bound ferritin WGA. (*d* and *e*) Tangential views of representative nuclear pores containing bound ferritin WGA. (*c*) A cross section of a representative nuclear pore in nuclei incubated with WGA plus chitotriose. (*f*) A tangential view of a representative nuclear pore of a nucleus incubated with WGA plus chitotriose. Bar, 100 nm.

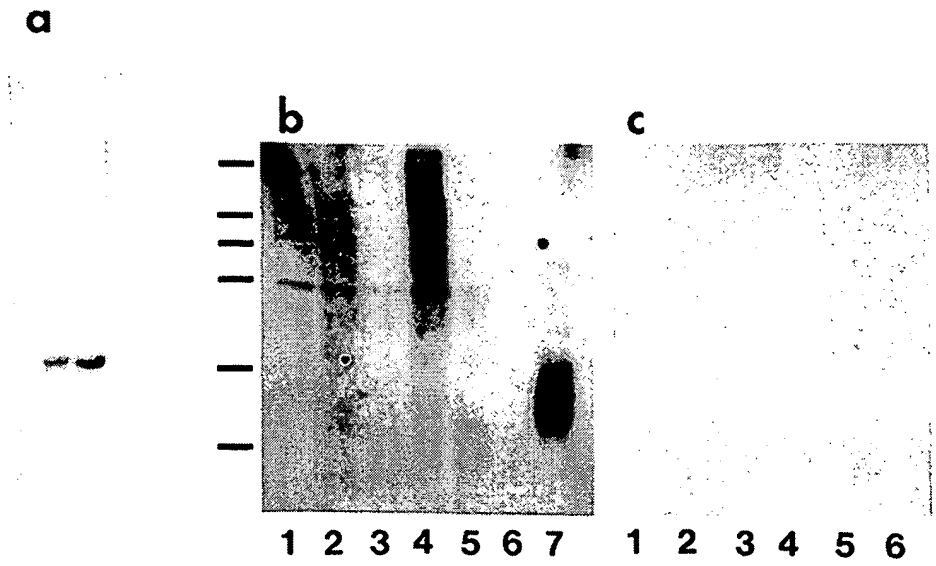

Figure 7. WGA-binding nuclear proteins. Proteins were separated on an SDS polyacrylamide gel, electrophoretically blotted onto nitrocellulose, and the blot probed with ^{125}I-labeled WGA. (*a*) Rat liver nuclear proteins of nuclei prepared with our usual buffers (*left lane*) and using the buffers of Blobel and Potter (1966) (*right lane*). (The protein aliquots resolved in the two lanes were not normalized for equal numbers of rat liver nuclei.) (*b*) The fractions probed were: egg extract, lane *1*; rat liver nuclei (1.5×10^6), lane *2*; rat liver nuclear pore complex–lamina fraction (1.25×10^6), lane *3*; supernatant of rat liver nuclei treated with 2% Triton X-100 (3×10^6), lane *4*; pellet of Triton-treated rat liver nuclei, lane *5*; and nucleoplasmin (5 μg), lane *6*. Lane *7* contained molecular mass markers, one of which is a WGA-binding glycoprotein (ovalbumin, 45 kD). The other markers were visualized by staining the blot with India ink. These are: myosin heavy chain (205 kD), beta-galactosidase (116 kD), phosphorylase B (97.4 kD), bovine plasma albumin (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). (*c*) No bands were observed when a blot identical to that in *b* was incubated with ^{125}I-WGA and 0.5 M *N*-acetylglucosamine. Lanes *1–6* are as in *b*.

complexes, and egg extract were resolved on an SDS polyacrylamide gel, transferred to nitrocellulose, and incubated with ^{125}I-WGA. Autoradiography of the blot revealed that rat liver nuclei contain one major protein of 63–65-kD that binds ^{125}I-WGA (Fig. 7 *a*; Fig. 7 *b*, lane *2*), as well as several minor ones of higher molecular mass. Co-incubation of an identical blot with 500 mM *N*-acetylglucosamine blocked the binding of ^{125}I-WGA to all these proteins, indicating a specific interaction between WGA and the sugar residues present on the proteins (Fig. 7 *c*, lanes *1–6*). A *Xenopus* WGA-binding glycoprotein co-migrating with the 63–65-kD rat protein was also found in the egg extract, as were other WGA-binding glycoproteins (Fig. 7 *b*, lane *1*).

The 63–65-kD band was only weakly detectable in blots of rat liver nuclear pore–lamina complexes probed with ^{125}I-labeled WGA (Fig. 7 *b*, lane *3*). Although the 63–65-kD band is similar in size to the lamin proteins, its depletion in the nuclear pore complex–lamina fraction, which is enriched in lamins, argues against it being a lamin protein. (An identical blot, incubated with antilamin A and C antisera, indicated our pore complex–lamina preparation to be ~5–10-fold enriched in lamin protein with respect to total rat liver nuclei; data not shown). As an additional control, nucleoplasmin itself was tested for binding of ^{125}I-labeled wheat germ agglutinin (Fig. 7 *b*, lane *6*) and found to have no affinity for the lectin, indicating that WGA does not inhibit transport by direct interaction with nucleoplasmin.

To investigate the possibility that the 63–65-kD glycoprotein is a nuclear membrane protein and/or a nuclear pore constituent, rat liver nuclei were treated with 2% Triton X-100 to remove all or part of the nuclear membranes (Aaronson and Blobel, 1974). The membrane fraction (Fig. 7 *b*, lane *4*) and the depleted nuclear fraction (Fig. 7 *b*, lane *5*) were electrophoresed on an SDS polyacrylamide gel, transferred to nitrocellulose, and incubated with ^{125}I-labeled WGA. The 63–65-kD glycoprotein, as well as most of the other nuclear glycoproteins, were removed by Triton treatment of the nuclei and were present in the membrane fraction (Fig. 7, lane *4*). Thus, the 63–65-kD glycoprotein is extractable by Triton and is likely to be either a nuclear membrane protein or an extractable nuclear pore protein. Our electron microscopy indicates that an abundant WGA-binding protein is a nuclear pore protein.

Discussion

In this report, we used an in vitro nuclear transport assay that faithfully mimics in vivo nuclear protein transport to screen for inhibitors of nuclear transport. We thus hoped to identify proteins involved in transport. It had been previously shown that RNA efflux from isolated nuclei was inhibited by millimolar concentrations of either of two lectins, Con A or WGA (Baglia and Maul, 1983). When we tested these and other lectins for binding to nuclei or inhibition of nuclear transport, we found that WGA both bound to nuclei and completely blocked nucleoplasmin transport.

Lectins have previously been observed to bind to nuclei. The lectin Con A interacts with a 180-kD glycoprotein shown to be a nuclear pore protein, the carbohydrate portion of which lies within the cisternal space of the nuclear membranes (Gerace et al., 1982). Ferritin Con A binds to the cisternal faces of both the inner and outer nuclear membranes, but not to the nucleoplasmic or cytoplasmic faces of the membranes (Virtanen and Wartiovaara, 1976; Feldherr et al., 1977; Virtanen and Wartiovaara, 1978; Schindler et al., 1985). FITC-Con A in our assay bound exclusively to damaged nuclei, presumably by gaining access to the cisternal space, and had no effect on nucleoplasmin transport, which occurs only in intact nuclei (Newmeyer et al., 1986*a*).

Fluorescent WGA has also been seen by others to stain

nuclei. These studies observed FITC-WGA at the nuclear periphery but did not further localize the WGA-binding sites (Nicolson et al., 1971; Virtanen and Wartiovaara, 1976; Seve et al., 1984; Schindler et al., 1985). The FITC-WGA staining that we observe is of a punctate nature. Because of the capacity of WGA molecules to self-agglutinate, a punctate pore staining pattern would not be distinguishable in the light microscope from the pattern that might result from WGA agglutination of non-pore-associated nuclear membrane glycoproteins. However, electron microscopy with ferritin WGA indicates that the fluorescent punctate pattern is pore-related. We found no evidence from electron microscopy for WGA-mediated agglutination of nuclear pores, although pores were often seen in loose clusters, even when competing sugar was present. Since the rat liver nucleus has been estimated to contain ~3–4,000 nuclear pores (Maul, 1977) and we observe fluorescent punctate entities numbering in the hundreds, the punctate staining pattern presumably represents naturally occurring clusters of nuclear pores.

The addition of WGA to our system resulted in a complete inhibition of nucleoplasmin transport. It appeared from the experiment involving preincubation of nuclei with WGA and subsequent washing that WGA caused transport inhibition by binding to a component of the nucleus itself, rather than an extract component. This inhibition was not seen in the presence of 500 mM *N*-acetyl-D-glucosamine, indicating that inhibition was the result of specific recognition of one or more glycoproteins by WGA. Furthermore, inhibition of accumulation by WGA was reversible upon later addition of a competing sugar, indicating that continued binding of WGA was necessary for inhibition and that inhibition could be reversed by dissociation of WGA from its binding site.

WGA is known to bind to terminal *N*-acetylglucosamine (GlcNAc) and sialic acid residues. Typically, these residues are indicators that a protein has passed through the Golgi and contains complex oligosaccharide chains. In the cell, such proteins are found within membrane-enclosed vesicles or organelles (Kornfeld and Kornfeld, 1976). Recently, however, several groups have identified a new type of glycoprotein containing simple GlcNAc monomers (Schindler and Hogan, 1984; Torres and Hart, 1984; Holt and Hart, 1986). Each monomer is attached to a protein by an O-linkage (Holt and Hart, 1986). Proteins with these residues are highly concentrated in nuclear and cytosolic fractions. The most abundant protein of this type in rat liver nuclei is a protein of molecular mass approximating that of the 63–65-kD protein we observe (Holt and Hart, 1986). Our finding that all rat liver nuclei stain with FITC-WGA, while only damaged nuclei stain with FITC-Con A, suggests that the WGA-binding GlcNAc residues reside on the exterior of the nucleus. Although this would be an unexpected location for a glycoprotein containing a complex-type oligosaccharide chain, the unusual subcellular distribution of glycoproteins with single O-linked GlcNAc residues (Schindler and Hogan, 1984; Holt and Hart, 1986) makes it entirely possible that nuclear proteins with single GlcNAc residues face outward into the cytoplasm. There is additional precedent for glycoproteins in regions of the cell other than the cisternal spaces of organelles; several groups have found glycoproteins in the interior of the nucleus (Hozier et al., 1980; Seve et al., 1984; Kan and Pinto da Silva, 1986). Clearly, our electron microscopic results indicate that WGA binding sites are present on the cytoplasmic face of each nuclear pore.

If every pore contained an equal amount of the WGA-binding protein, the amount of fluorescent WGA binding could be used as an estimate of pore number for individual nuclei. If this were true, the newly added portion of hybrid rat liver nuclear envelopes or of regrown sperm nuclear envelopes must have fewer pores per unit area, since they stain much more faintly with FITC-WGA. An alternate explanation, however, for the fainter staining of some nuclear envelopes could be that individual nuclear pores vary in the amount or glycosylation level of WGA-binding protein they contain. Theoretically, variation in a protein involved in pore function might either regulate the state of the pore (i.e., open or closed) or affect the rate of transport through the pore. We find that the WGA-binding pore protein does affect pore function, at least when bound to WGA, and does vary in nuclei from different sources. We further find that, when intermediate concentrations of WGA are used in the transport assay, the same number of nuclei are active in transport, but the amount of TRITC nucleoplasmin observed per nucleus decreases as the concentration of WGA increases. Unfortunately, this latter result does not let us distinguish between (*a*) induction of an off state in an increasing number of pores and (*b*) a gradual slowing of the rate of transport through each pore as more WGA binds to the pore.

In a recent report, WGA was found to have no effect on the influx of a 64-kD fluorescent dextran into the nucleus (Jiang and Schindler, 1986). In support of this finding, we see no exclusion of 10- and 20-kD fluorescent dextrans by WGA in experiments where nucleoplasmin is excluded. Similarly, we see no difference in the largely non-nuclear distribution of 40-, 70-, and 150-kD FITC-labeled dextrans when WGA is present in our system (observations recorded at 30 min–3 h; Finlay, D., and D. Forbes, unpublished). Since WGA blocks nucleoplasmin transport, it appears that this nuclear protein enters the nucleus by a pore interaction different from that of the non-nuclear dextran molecule.

In investigating the target of action of WGA, we found that WGA bound strongly to one major (63–65-kD) and several minor rat liver nuclear proteins. The 63–65-kD protein is depleted from rat liver pore complex-lamina fractions (relative to the total nuclear membrane fraction) and is thus not a lamin protein. Although we have not presented direct evidence that the 63–65-kD protein is the target of inhibition of transport by WGA, it is strikingly the most abundant WGA-binding protein in rat liver nuclei. Our data clearly implicate at least one glycoprotein in the nucleus which, when bound by WGA, inhibits the transport of nucleoplasmin. In addition, our observation that ferritin-labeled WGA binds to pores demonstrates that there are WGA-binding glycoproteins in nuclear pores and that these glycoproteins are present in multiple copies. At the completion of this work, Davis and Blobel (1986) reported that a 62-kD protein, identified by a monoclonal antibody, is located in nuclear pores. Their work also indicates that there are several molecules of the 62-kD protein in the pore, and that, when isolated, this protein binds WGA. Thus it is likely that this protein and the 63–65-kD protein described here are the same, and, moreover, that the 63–65-kD protein is a target of WGA inhibition. We are presently testing our supposition that the 63–65-kD protein, when bound by WGA, inhibits nuclear protein transport by the production of antibodies to the 63–65-kD protein.

Possible explanations for the inhibition of nuclear transport by WGA would place the 63–65-kD (or other) glycopro-

tein in the nuclear pore. WGA when bound to the glycoprotein might either: (*a*) physically block the pore so that large nucleoplasmin molecules could no longer pass through, (*b*) bind to a recognition signal of the pore glycoprotein that is required for the binding and subsequent transport of nucleoplasmin, or (*c*) alter the glycoprotein so that pore function is destroyed. Our preliminary result that WGA does not interfere with the passage of fluorescent dextrans into the nucleus argues against a total blockage of the pore by WGA. In any event, the ease and specificity with which WGA inhibition of transport is reversed by subsequent sugar addition argue that the pore is not permanently altered by WGA.

In summary, the results reported here describe the first identified inhibitor of nuclear protein transport, the lectin WGA. We have shown that WGA completely blocks nuclear transport and binds directly to the nuclear pore. A pore glycoprotein thus appears to be either directly involved in nuclear transport or to be placed in such a position that WGA can, by binding to it, obstruct the passage of nuclear proteins through the pore. The most likely candidate for the target of WGA binding is the 62-kD pore glycoprotein recently observed by Davis and Blobel (1986) and independently by us as the major WGA-binding protein (63–65-kD) in rat liver nuclei. We hope by extending these studies to further probe the structure and function of the nuclear pore.

The authors thank Kathy Wilson, William Dunphy, and John Newport for critical reading of the manuscript. We also thank Scott Lonergan and Madhu Singh for help with the figures.

This work was supported by a grant to D. Forbes from the National Institutes of Health (GM-33279). D. Forbes is the recipient of a Pew Scholarship in the Biomedical Sciences.

Received for publication 15 July 1986, and in revised form 20 October 1986.

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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14394–14399, December 1997
Biochemistry

Inhibition of mRNA export in vertebrate cells by nuclear export signal conjugates

(protein kinase inhibitor/REV/exportin 1)

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Contributed by James E. Dahlberg, October 20, 1997

ABSTRACT **Leucine-rich nuclear export signals (NESs) are recognized by the NES receptor exportin 1 and are central to the export of multiple shuttling proteins and RNAs. The export of messenger RNA in vertebrates was, however, thought to occur by a different pathway, because inhibition by injection of a synthetic Rev NES conjugate could not be demonstrated. Here we find that peptide conjugates composed of the NES of either protein kinase A inhibitor protein (PKI) or the HIV-1 Rev protein, when coupled to human serum albumin, are potent inhibitors of mRNA and small nuclear RNA export. These results provide direct evidence that mRNA export in vertebrates depends on interactions between an NES and its cognate NES receptors. PKI NES conjugates are significantly more efficient at inhibiting RNA export than are REV NES conjugates, indicating that different NESs may have different abilities to promote protein and RNA export. Surprisingly, an expected control conjugate containing the mutant Rev NES sequence M10 strongly inhibited the export of intronless dihydrofolate reductase mRNA. Nuclear injection of NES peptide conjugates led to mislocalization to the nucleus of 10–20% of the cytoplasmic Ran GTPase-binding protein (RanBP1) indicating that RanBP1 shuttles between the nucleus and the cytoplasm via an NES pathway. These results demonstrate that in vertebrates the export of mRNA, like that of small nuclear RNA, 5S rRNA, and transport factors such as RanBP1, employs NES-mediated molecular machinery.**

Nuclear export of several proteins is directed by nuclear export signals (NESs) that consist of short amino acid sequences rich in leucine or other aliphatic amino acids plus acidic residues. Whereas NES is a general term for a nuclear export signal, throughout this paper NES refers specifically to the class of leucine-rich NESs, and peptides containing these sequences are named in uppercase letters. Such signals were first identified in the Rev protein of the HIV type 1 (HIV-1) and in the protein kinase A inhibitor protein (PKI) (1–6). Similarly, two yeast proteins that are essential for export of poly(A)$^+$ RNA, Gle1p and Mex67p, also contain functional NES sequences (7, 8).

In yeast two-hybrid experiments, NESs have been shown to interact, perhaps indirectly, with a variety of proteins such as the Rev-interacting proteins (hRip/Rab) (9–12) and several yeast and vertebrate nucleoporins (nuclear pore proteins; refs. 11, 13, 14). In vertebrates, at least one nucleoporin, Nup98, plays an important role in the export of mRNAs, small nuclear RNAs (snRNAs), 5S rRNA, and large rRNAs (15), and two others, Nup153 and Nup214/CAN, may be involved in the export of poly(A)$^+$ RNAs (16, 17).

NES-mediated export is saturable and therefore dependent on one or more limiting cell factors (3, 18). By coupling many copies of the Rev NES peptide CLPPLERLTL onto BSA, Fischer *et al.* (3) generated an inhibitor of RNA export; as a control they used a mutant form of Rev NES, M10 (1, 19). Injection of these conjugates into *Xenopus laevis* oocyte nuclei showed that the Rev NES conjugate inhibited Rev–RRE-mediated RNA export (RRE, Rev response element), as well as snRNA and 5S rRNA export. This inhibition was attributed to titration of a putative NES receptor. However, their Rev NES conjugate did not cause inhibition of mRNA export; this finding is surprising because several yeast proteins involved in mRNA export contain NES sequences essential for their action. More recently, a conjugate containing the NES of the yeast protein Mex67p was also reported to inhibit the export of snRNAs and 5S RNA, but not mRNA (8). The lack of effect of these NES conjugates on the export of mRNA was interpreted as indicating that mRNA uses a factor with a signal that is recognized by a different type of receptor.

Recent work has identified the highly conserved protein CRM1 as a major receptor for the NESs of Rev and PKI (and by implication for the NES of other proteins) in both yeast and vertebrates (12, 20, 21). The CRM1 protein, renamed exportin 1, forms a complex with the NES of a "cargo" protein, but does so only in the presence of RanGTP (20), the nuclear form of the Ras-like GTPase Ran (22). This NES cargo–exportin 1–RanGTP trimeric complex is thought to interact directly or indirectly with proteins of the nuclear pore, to facilitate translocation of the NES cargo through the nuclear pore (reviewed in ref. 23). In one study, exportin 1 was found to be required for the export of poly(A)$^+$ nuclear RNAs in yeast (21), making it curious that conjugates containing the Rev NES failed to inhibit the export of mRNA in vertebrates (3).

To analyze the role of NES in RNA export more closely, we tested conjugates containing the NES from PKI for their abilities to affect export from *Xenopus* oocyte nuclei. Surprisingly, we found that the PKI conjugate strongly inhibited the export of mRNA, in addition to snRNA. This led us to test Rev NES conjugates for comparison, and we found that Rev NES conjugates also inhibited mRNA export, albeit less well than PKI NES conjugates. We find a hierarchy of inhibition, ranging from a very strong inhibition by PKI NES to a moderate inhibition by our Rev NES conjugates, with even the Rev mutant NES M10 conjugate inhibiting export to a slight but

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Abbreviations: NES, nuclear export signal; PKI, protein kinase A inhibitor protein; RanBP1, Ran-binding protein 1; snRNA, small nuclear RNA; RRE, Rev response element; DHFR, dihydrofolate reductase; AdML, adenovirus major late; HSA, human serum albumin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; RanGAP, Ran GTPase-activating protein.

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Table 1. Amino acid content of peptide conjugates

Conjugate	Peptide
PKI	CNELALKLAG LDINKT
MIX	CTKNILDGALKLALNE
REV	CLPPLER LTLD
BSA-R	CLPPLER LTL
M10	CLPPDLR LTLD

Peptides PKI, MIX, REV, and M10 were conjugated to HSA and used in injection experiments as described in the text. BSA-R refers to the Rev-NES peptide conjugate used by Fischer *et al.* (3). Leucine residues shown to be critical for function of the nuclear export signals (1, 2, 4) are in bold. Mix is a scrambled PKI NES.

significant degree. A scrambled PKI NES sequence gave no inhibition of export. Nuclear injection of the normal PKI NES conjugate also led to accumulation in the nucleus of the NES-containing Ran-binding protein 1 (RanBP1), raising the possibility that mislocalization of this normally cytoplasmic Ran-binding protein contributes to the persistent inhibition of RNA export that we observe at later time points.

MATERIALS AND METHODS

DNA Templates and *in Vitro* Transcription. DNA templates for transcription of U3 (24), $U1_{Sm-}$ (25), and U5 snRNAs (26) were generated by PCR amplification of RNA coding regions by using appropriate primers as described previously. The templates for transcription of dihydrofolate reductase (DHFR) mRNA (an intronless mRNA; ref. 27), pre-adenovirus major late (AdML) mRNA (an adenovirus intron-containing pre-mRNA substrate; refs. 15 and 28), and $tRNA_i^{Met}$ (ref. 29; E.L. and J.E.D., unpublished data) were generated by linearization of previously described plasmids. *In vitro* transcription of radiolabeled RNA was performed as described (26, 30) by using [α-^{32}P]GTP and SP6 RNA polymerase (for U3, $U1_{Sm-}$, U5, and pre-AdML RNAs) or T7 RNA polymerase (for DHFR mRNA and tRNA) according to manufacturer's conditions (Promega). All RNAs were synthesized with m^7GpppG caps (25), except for tRNA, which was made as uncapped RNA.

Peptide Conjugate Preparation. Peptides of the sequences given in Table 1 were synthesized, and their structures were confirmed by mass spectroscopy (Research Genetics, Huntsville, AL). A cysteine was included at the N terminus of each peptide for use in crosslinking. For preparation of conjugates, human serum albumin (HSA; Calbiochem) was dissolved in 0.1 M sodium bicarbonate, pH 8.5 (conjugation buffer 1), and mixed with the bifunctional crosslinker, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Pierce) (10 mg/ml in dimethylformamide), to give final concentrations of 10 mg/ml HSA and 2.5 mg/ml MBS. Following a 1-hr incubation at room temperature, the free crosslinker was removed by using a Bio-Gel P6-DG column (Bio-Rad) equilibrated in 0.1 M sodium phosphate, pH 6.0 (conjugation buffer 2). HSA/MBS was pooled, and 5 mg was mixed with 2.5 mg of peptide (10 mg/ml in conjugation buffer 2) and incubated for 1 hr at room temperature. Free peptide was removed by using a Bio-Gel P6-DG column equilibrated in phosphate-buffered saline. Conjugate-containing fractions were pooled and adjusted to the indicated concentrations by using a Microcon 30 concentrator (Amicon). For production of biotinylated conjugates, HSA (10 mg in 1 ml of conjugation buffer 1) was first incubated with 0.4 mg of *N*-hydroxysuccinimide-LC-biotin II (Pierce) for 1 hr at 4°C. Biotinylated HSA was separated from free biotin by several rounds of concentration with a Centriprep 10 (Amicon). Peptide conjugation was then performed as described above. The number of peptides per conjugate was estimated following gel electrophoresis and comparison with HSA/MBS. Protein concentrations were determined by the Bio-Rad protein assay. Once conjugated to HSA, the peptide sequences of the REV, M10, and PKI peptides (Table 1) were again confirmed by 10 rounds of Edman sequencing on an Applied Biosytems Procise Microsequencer.

Oocyte Injections and Analysis of RNA and Protein Transport. Preparation and injection of *X. laevis* oocytes were performed as previously described (24–26). To analyze the effect of peptide conjugates on the export of specific RNAs, 1–5 fmol of individual ^{32}P-labeled RNAs was mixed with the peptide conjugates in 9 mM NaCl/0.6 mM DTT/0.25 unit/μl RNasin and injected in the amounts indicated in the figure legends. The mixtures were injected into the nuclei of oocytes, and at the times indicated in the figure legends, the oocytes were dissected under oil (31). The nucleocytoplasmic distributions of the RNAs were analyzed by polyacrylamide gel electrophoresis, as previously described (26, 32). U3 snRNA, which normally is not exported from the nucleus (24), and blue dextran (29) were included in all injection experiments as controls for the accuracy of nuclear injection and oocyte dissection.

To analyze the effects on the intracellular localization of endogenous transport factors, the peptide conjugates were injected into the nuclei of oocytes in the amounts indicated in the legend to Fig. 4. The oocytes were subsequently dissected into nuclear and cytoplasmic fractions, and the nuclear proteins were solubilized directly in 1× SDS gel loading buffer. Cytoplasms were homogenized in 10 mM Mops, pH 7.2/75 mM KCl/25 mM NaCl/2 mM DTT/1 μg/ml leupeptin/1 μg/ml pepstatin/2 μg/ml aprotinin. After removal of the yolk from the cytoplasmic extract by centrifugation at 14,000 rpm in a microcentrifuge for 4 min, the cleared extract was mixed 1:1 with 2× SDS gel loading buffer before resolution by gel electrophoresis. Proteins from the nuclear and cytoplasmic fractions, as indicated in the legend to Fig. 4, were separated in SDS-containing 10% polyacrylamide gels (33). The distributions of specific proteins were analyzed by immunoblotting by using appropriate antibodies to the different transport factors and ECL according to the manufacturer's protocol (Amersham). Rabbit polyclonal antibodies were the generous gifts of M. Dasso (National Institute of Child Health and Human Development, Bethesda, MD) [anti-Ran GTPase-activating protein (RanGAP), anti-Ran, and anti-RanBP1], M. Moore (Baylor College of Medicine, Houston, TX) (anti-importin β), F. Grosveld (St. Jude's Hospital, Memphis, TN) [anti-CRM1 (exportin 1)], and D. Görlich (ZMB Universität, Heidelberg, Germany) (anti-importin α) and were used for immunoblotting at the following dilutions: anti-Ran, 1:1,000; anti-RanGAP, 1:2,000; anti-RanBP1, 1:2,000; anti-importin α, 1:10,000; anti-importin β, 1:750; and anti-CRM1, 1:2,000.

RESULTS

Inhibition of mRNA Export by a PKI NES Conjugate. We asked whether a PKI NES conjugate could competitively inhibit the export of snRNA from oocyte nuclei, as had been previously reported for Rev and Mex67p NES conjugates (3, 8). PKI NES peptides conjugated to HSA (Table 1) did indeed inhibit the export of U1 snRNA (Fig. 1, lanes 2–7). Surprisingly, the PKI conjugate was also a very effective inhibitor of the export of DHFR mRNA and spliced AdML mRNA (lanes 2–7). The export of tRNA was unaffected by the PKI conjugate, demonstrating that the inhibition was specific. A control conjugate, containing a peptide with the same amino acids as PKI NES, but arranged in a mixed order that does not resemble an NES sequence (termed MIX conjugate; Table 1) had no effect on the export of any class of RNA (Fig. 1, lanes 8–13).

The amount of PKI conjugate used in the experiments shown above (≈20 ng per nucleus) was low, only about one-seventh as much as Fischer *et al.* (3) used in studies with their Rev NES conjugate (designated BSA-R in Table 1). To compare the NES of PKI and Rev more closely, we synthesized a Rev NES peptide (designated REV in Table 1); this conjugated peptide contains at its C terminus an aspartic acid residue that is not present in BSA-R but is present in Rev protein. When a low amount of our REV conjugate (≈20 ng) was injected into oocytes, very little inhibition of U1 RNA or mRNA export was observed (Fig. 1, lanes 14–19),

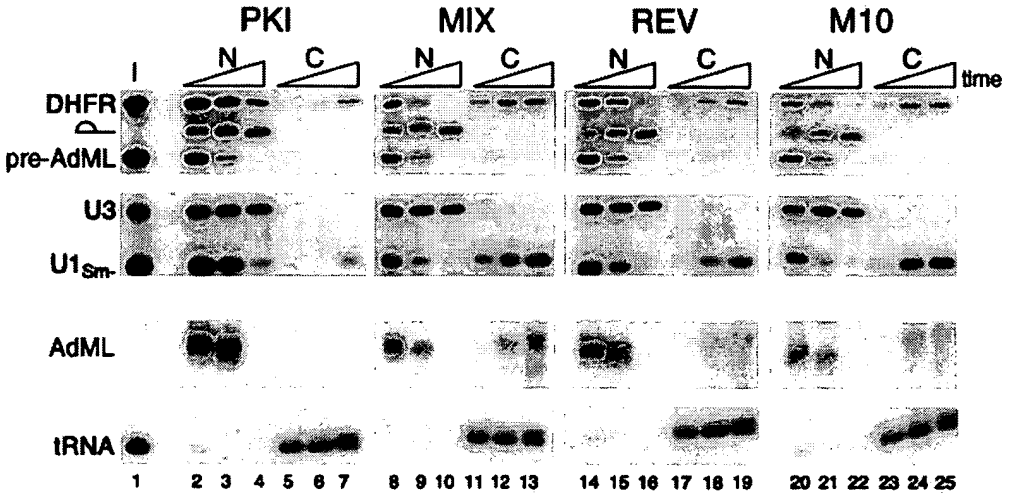

FIG. 1. Inhibition of mRNA and snRNA export by the PKI NES conjugate. The PKI and REV NES conjugates differ in their abilities to inhibit RNA export. One to three femtomoles of each ^{32}P-labeled DHFR mRNA, AdML pre-mRNA, U3 snRNA, U1$_{Sm-}$ snRNA, and tRNA$_i^{Met}$ were coinjected into oocyte nuclei together with ≈20 ng per nucleus of the PKI NES (lanes 2–7), MIX (lanes 8–13), REV NES (lanes 14–19), or M10 (lanes 20–25) peptide conjugates (see Table 1). The oocytes were dissected 1, 4, and 20 hr after injection, and RNA was extracted from the nuclear (N) and cytoplasmic (C) fractions; 0.5 oocyte equivalent of RNA was resolved on denaturing 8% polyacrylamide gels and visualized by autoradiography. The AdML mRNA and tRNA$_i^{Met}$ panels were exposed twice as long as the upper panels, to allow for detection of AdML mRNA export. The triangles at the top of the lanes indicate increasing times since injection. U3 snRNA, which normally is not exported from the nucleus (24), served as a marker for the accuracy of nuclear injections and oocyte dissections. The RNA injection mixture (I) is shown in lane 1. Pre-AdML RNA is the injected form of the intron-containing pre-mRNA; the lariat symbol represents the excised AdML intron, and AdML represents the spliced mRNA, which normally is exported from the nucleus.

consistent with the previous study of Fischer *et al.* (3). Only at early time points were the levels of exported U1 RNA different in oocytes that had been injected with the REV conjugate vs. a mutant REV conjugate, M10 (Table 1), in which two amino acids were changed (Fig. 1, lanes 20–25). Thus, the PKI conjugate is a much better inhibitor of RNA export than is the REV conjugate. This same low level of the PKI conjugate was also a very effective inhibitor of RNA export mediated by Rev-RRE (data not shown), indicating that the PKI conjugate most likely titrates export factors utilized by the Rev NES. Finally, even the low level of the PKI conjugate used in Fig. 1 was in excess over what was needed to interfere with RNA export because injection of 5- to 10-fold lower amounts of the PKI conjugate retarded the export of both snRNAs and mRNAs (data not shown).

Inhibition of mRNA Export by a REV NES Conjugate. When higher amounts of our REV conjugate were injected at a level

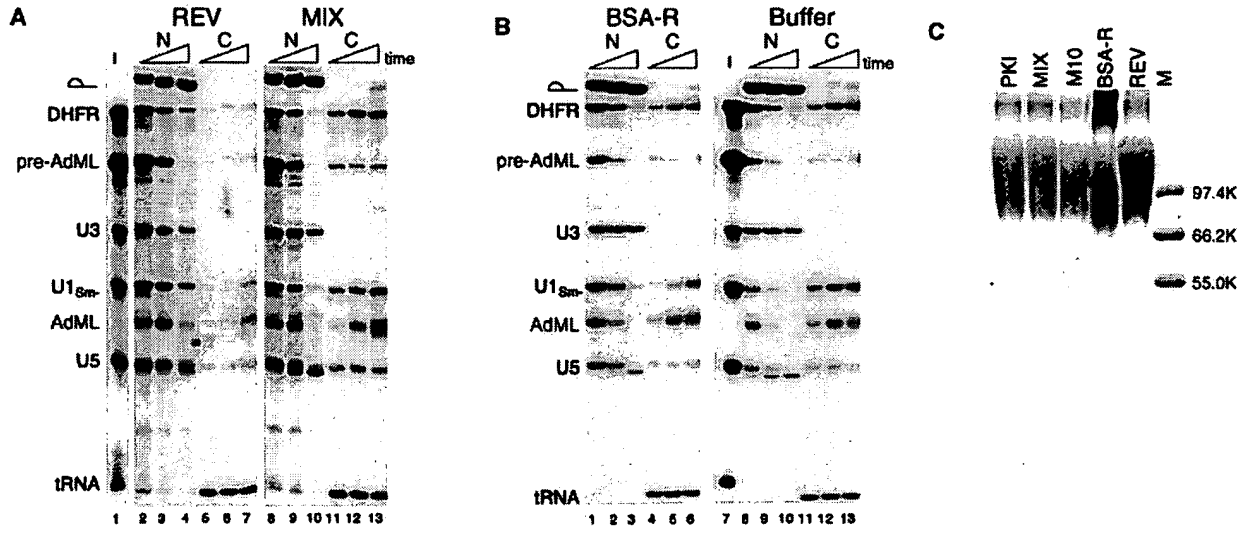

FIG. 2. Inhibition of mRNA export by the REV NES conjugate. (*A*) The REV NES conjugate inhibits mRNA and snRNA export when present in high amounts. Mixtures containing ^{32}P-labeled DHFR mRNA, AdML pre-mRNA, U3 snRNA, U1$_{Sm-}$ snRNA, U5 snRNA, and tRNA$_i^{Met}$ (I, lane 1) were coinjected into oocyte nuclei together with ≈130 ng per nucleus of the REV (lanes 2–7) or the MIX (lanes 8–13) peptide conjugates. Oocytes were dissected 1.5, 4, and 21 hr after injection, and RNA export was analyzed as in Fig. 1. The export of a small amount of pre-AdML RNA is because of saturation of the splicing machinery by the bolus of injected RNA. (*B*) The BSA-R conjugate fails to inhibit mRNA export even when present in high amounts. Mixtures containing ^{32}P-labeled DHFR mRNA, AdML pre-mRNA, U3 snRNA, U1$_{Sm-}$ snRNA, U5 snRNA, and tRNA$_i^{Met}$ (I, lane 7) were coinjected into oocyte nuclei together with ≈190 ng per nucleus of the BSA-R (lanes 1–6) conjugate or with buffer alone (lanes 8–13). Oocytes were dissected 1.5, 4, and 18.5 hr after injection, and RNA export was analyzed as in Fig. 1. (*C*) SDS/PAGE analysis of peptide conjugates. Ten micrograms of each of the indicated peptide conjugates (Table 1) were subjected to electrophoresis in an SDS-containing 8% polyacrylamide gel, and the proteins were detected by Coomassie blue staining. The marker (M) lane contains 0.5 μg of each protein molecular weight marker (Promega).

comparable with that used by Fischer *et al.* (3) (≈130 ng per nucleus), the export of snRNAs was effectively blocked (U1 and U5; Fig. 2*A*, lanes 2–7), as had been observed previously. However, our Rev conjugate also inhibited the export of both DHFR mRNA and spliced AdML mRNA (compare REV to MIX, Fig. 2*A*). The REV conjugate did not affect export of tRNAs, again showing that the inhibition of export was specific. Thus, both the PKI conjugate and the REV conjugate that we tested blocked export of mRNAs, as well as snRNAs. However, the REV conjugate was less potent, requiring a higher injected concentration for export inhibition. Because these results differed from those of Fischer *et al.* (3) with regard to inhibition of mRNA export, we repeated our experiments by using BSA-R, a REV NES conjugate provided by U. Fischer (IMT, Philipps-Universität, Marburg, Germany). In that case, we obtained results identical to those previously reported by him for this conjugate: inhibition of snRNA export at early time points, relief of inhibition of snRNA export at late time points, and no inhibition of mRNA export (Fig. 2*B*, lanes 1–6). Thus, the conjugates used by Fischer *et al.* and by us differ in activity.

Compositions of the Conjugates. We questioned how the preparations of Rev NES conjugates used by Fischer *et al.* (3) and by us might differ. The presence of biotin on our HSA carrier protein is unlikely to be significant, because control experiments performed with biotin-free REV conjugate gave similar results (data not shown); moreover, the MIX conjugate, which was biotinylated, did not inhibit export even when injected at high concentrations (Fig. 2*A* and Fig. 3*B*). Previous studies have shown that the presence of biotin does not affect the export of conjugates from vertebrate nuclei (18). The use of HSA, rather than BSA, as the carrier protein is not the critical difference, because the MIX-HSA conjugate has no effect on export. Sequencing of our REV and M10 peptide conjugates confirmed that their sequences were as presented in Table 1 (data not shown). One obvious difference common to both our REV conjugate and M10 conjugate is the presence of an aspartic acid residue at the C terminus of the peptide on each conjugate (Table 1). However, when we resynthesized and tested the REV and M10 peptides in the form of nonbiotinylated conjugates with or without this aspartic acid, we observed no detectable difference in the pattern of RNAs whose export was inhibited, although there was a modest but reproducible decrease in the ability of the shorter conjugates to inhibit RNA export (data not shown).

It is possible that the conditions under which the peptides were conjugated to the protein carriers are responsible for the differences in the abilities of the conjugated proteins to inhibit mRNA export. We carried out peptide conjugation for 1 hr, whereas Fischer *et al.* (3) carried out the peptide conjugation reaction for *ca.* 16 hr. By polyacrylamide gel electrophoresis (Fig. 2*C*), we estimate that our conjugates and the BSA-R conjugate furnished to us by Fischer have about the same average number of peptides per carrier (≈15–20). However, the electrophoretic profiles of the two conjugates indicated that a significant proportion of the BSA-R conjugate is in very large complexes, possibly reflecting extensive crosslinking between the conjugates.

Inhibition of DHFR mRNA Export by the M10 Conjugate. When comparing the inhibitory activities of the different NES conjugates relative to their controls, we noted a difference between the M10 and MIX control conjugates. The M10 conjugate itself, even when injected at the relatively low amount in Fig. 1, appeared to inhibit export of DHFR mRNA slightly (Fig. 1, compare lanes 8–13 and 20–25). Therefore, we asked whether the M10 conjugate, at higher levels, might interfere with RNA export. Indeed, at higher concentrations, M10 conjugate strongly inhibited the export of DHFR mRNA and, to a lesser extent, the export of spliced AdML mRNA and snRNAs (Fig. 3*A*, lanes 1–6 compared with lanes 7–12); however, this M10 conjugate did not block Rev-mediated export of an RRE-containing RNA (data not shown). In contrast, even high amounts of the control MIX conjugate had no

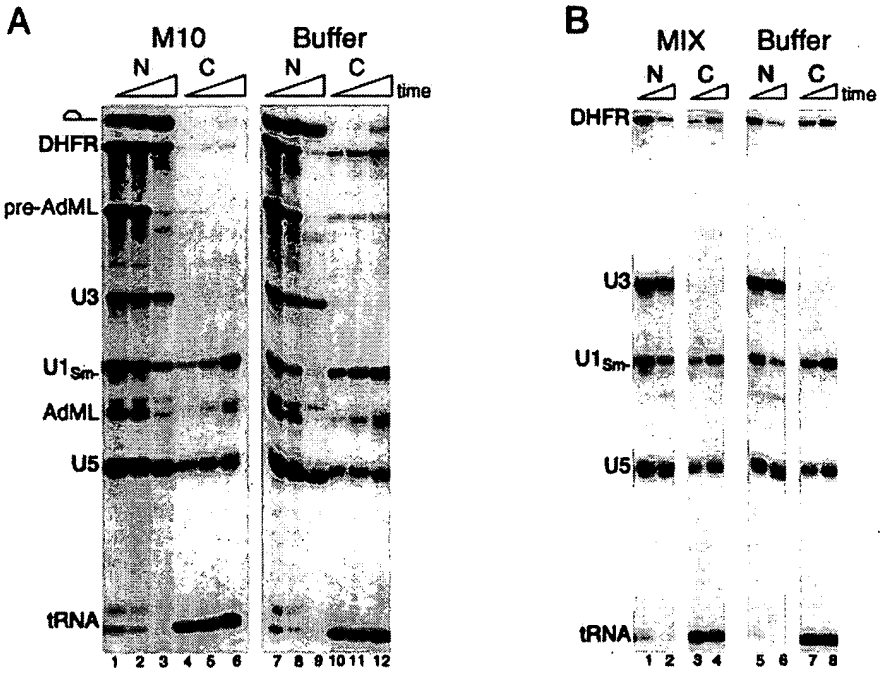

FIG. 3. The M10 but not the MIX peptide conjugate can inhibit RNA export. (*A*) The M10 conjugate is a potent inhibitor of DHFR mRNA export. Mixtures containing ^{32}P-labeled DHFR mRNA, AdML pre-mRNA, U3 snRNA, U1$_{Sm-}$ snRNA, U5 snRNA, and tRNA$_i^{Met}$ were coinjected into oocyte nuclei with ≈130 ng per nucleus of the M10 (lanes 1–6) peptide or with buffer only (lanes 7–12). Oocytes were dissected 1.5, 4, and 18 hr after injection, and RNA export was analyzed as in Fig. 1. (*B*) The MIX conjugate does not interfere with RNA export, even when present in high amounts. Mixtures containing ^{32}P-labeled DHFR mRNA, U3 snRNA, U1$_{Sm-}$ snRNA, U5 snRNA, and tRNA$_i^{Met}$ were coinjected into oocyte nuclei with ≈130 ng per nucleus of the MIX (lanes 1–4) peptide or with buffer alone (lanes 5–8). Oocytes were dissected 1.5 and 4 hr after injection, and RNA export was analyzed as in Fig. 1.

effect on RNA export when compared with injected buffer alone (Fig. 3*B*).

Protein and RNA Import in the Presence of the PKI NES. To test whether the efficient and broad inhibition of mRNA and snRNA export by the PKI NES conjugate reflected nonspecific effects on nuclear pore function, we monitored protein import from the cytoplasm in the presence of the conjugate. As expected, the PKI conjugate caused only a slight reduction in the rate of import of radioactively labeled nuclear proteins that had been injected into the cytoplasm (data not shown).

Although the import of proteins does not appear to be inhibited by the NES conjugates, the import of certain RNAs was significantly retarded. Injection of the PKI conjugate into the nucleus inhibited by ≈2- to ≈3-fold the rate of import of both U6 RNA and NL-15 RNA, an RNA whose import into the nucleus requires interaction with the nuclear antigen La (34) (data not shown). However, the PKI conjugate did not affect the import of U1 or U5 small nuclear ribonucleoproteins into the nucleus, which has been proposed to occur by a pathway different than that used by U6 small nuclear ribonucleoproteins (35, 36). The results reported here suggest that import of U6 and NL-15 RNAs depends on the presence of shuttling proteins whose export from the nucleus to the cytoplasm is affected by the NES conjugates (37).

Nuclear Mislocalization of RanBP1. To examine whether endogenous transport factors might be sequestered in the nucleus as a consequence of competition for NES receptors by the NES conjugates, we analyzed the intracellular distribution of the transport-related factors Ran, RanBP1, RanGAP, importin β, importin α, and exportin 1 by immunoblotting. For the majority of the proteins, no significant change in the nucleocytoplasmic distribution was evident (Fig. 4*A*). However, ≈10–20% of the RanBP1 was observed to accumulate in the nucleus in the presence of the PKI or REV conjugates but not in the presence of the MIX or M10 conjugates. This result supports the proposal that RanBP1, which has an essential NES, normally cycles between the nucleus and cytoplasm (38, 39), as blockage of its export by the NES conjugate would result in its accumulation in the nucleus. Aberrant localization of RanBP1 protein was observed for at least 19 hr after injection of the PKI conjugate (Fig. 4*B*), consistent with the persistent inhibition of RNA export (Fig. 1). In contrast, at this same time point we detected no significant nuclear accumulation of RanBP1 in oocytes injected with BSA-R; that may account for the eventual alleviation of inhibition of snRNA export by this conjugate (Fig. 2*B*; ref. 3).

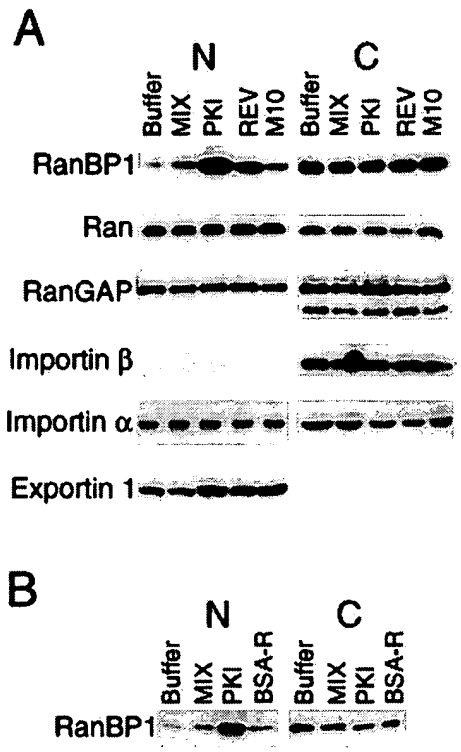

FIG. 4. RanBP1 mislocalizes to the nucleus in the presence of NES peptide conjugates. (*A*) The PKI and REV NES peptide conjugates affect the intracellular distribution of RanBP1 but not other tested components of the transport machinery. The indicated peptide conjugates (≈115 ng per nucleus) were injected into oocyte nuclei, and 6.5 hr later the oocytes were dissected. The protein extracts were separated in SDS-containing 10% polyacrylamide gels, and the distributions of specific proteins were analyzed by immunoblotting by using appropriate antibodies and ECL. Oocyte equivalents of the nuclear (N) and cytoplasmic (C) extracts varied for the different proteins: RanBP1, importin β, importin α, and exportin, 3.0 (N) and 0.1 (C); Ran, 1 (N) and 0.5 (C); and RanGAP, 0.5 (N) and 0.1 (C). (*B*) The PKI NES conjugate, but not the BSA-R conjugate, causes prolonged mislocalization of RanBP1. Oocyte nuclei were each injected with ≈130 ng (MIX and PKI) or ≈190 ng (BSA-R) of peptide conjugates or buffer. Nineteen hours later the oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions. The distribution of RanBP1 in 2 (N) and 0.1 (C) oocyte equivalents was analyzed by immunoblotting as in *A*.

DISCUSSION

The export of RNAs from the nucleus is believed to be mediated by specific RNA binding proteins (reviewed in ref. 40). A well known example of such a protein is the HIV factor Rev, in which a specific NES sequence has been characterized (reviewed in ref. 41). Similar leucine-rich NES sequences have been found in PKI and in several factors implicated in poly(A)$^+$ RNA export in yeast. A potential NES receptor, exportin 1, has recently been identified in both yeast and higher eukaryotes (reviewed in ref. 23).

Peptides consisting of an NES sequence coupled to a carrier protein such as serum albumin act as competitive inhibitors of Rev-mediated export and of cellular snRNA and 5S rRNA export, presumably by titration of an NES receptor. The inhibition of export of several RNAs—but not of mRNA—by the REV NES conjugate used by Fischer *et al.* (3) led to the proposal that distinct signals and receptors are used for the export of the different classes of RNA. Specifically, they proposed that Rev, via its NESs, makes use of a receptor normally used for the export of snRNAs and 5S rRNA but that mRNA uses a different system (3). The recent identification of exportin 1 as a receptor for Rev NES and PKI NES (20, 21) is not inconsistent with this possibility. However, export of poly(A)$^+$ RNA in yeast depends on NES-containing proteins as well as exportin 1 and related proteins (7, 8, 42), making it likely that similar factors also function in the export of mRNAs in higher eukaryotes. Paradoxically, before this report no NES conjugate had been shown to be capable of inhibiting mRNA export in vertebrates (3, 8).

The experiments presented here show that in higher eukaryotes mRNA export is effectively blocked by competition with NES conjugates containing either PKI or REV NES. It is unclear why this inhibition of mRNA export by NES conjugates has escaped notice until now. Possibly, most experiments of this type used conjugates that were much more extensively crosslinked, as a consequence of carrying out the conjugation reaction for 16–18 hr rather than the 1 hr used here (see *Materials and Methods*). The extra time of incubation with the crosslinking reagent apparently allowed for formation of a significant proportion of larger aggregates between several protein molecules (Fig. 2*C*), which might differ in their interactions with NES receptors or receptor complexes.

Our REV conjugate and the BSA-R conjugate used by Fischer *et al.* (3) differ from each other functionally in their abilities to inhibit mRNA export, although the NES peptides differ chemically only by the presence of the aspartic acid residue in our conjugate (Table 1). This extra residue cannot be responsible for the difference in activities because we resynthesized a new peptide conjugate identical in amino acid sequence to the peptide in

BSA-R and found that this minimal Rev NES conjugate inhibited both snRNA and mRNA export (data not shown). Regardless of the differences between the REV and BSA-R conjugates, the fact remains that the REV NES conjugate inhibits the export of mRNA, as well as snRNA.

Whereas Rev is a bifunctional export factor that binds directly to RNA and to exportin 1, it is possible that comparable cellular mRNA export factors have these functions on separate polypeptides. Thus, an RNA binding protein may gain access to exportin 1 or a similar receptor through protein–protein interactions with an NES-containing protein. Therefore the factor(s) responsible for cellular mRNA export are not necessarily bifunctional RNA binding proteins that contain an NES (7).

Unexpectedly, we found that the M10 conjugate could also inhibit RNA export to some extent, being most effective on DHFR mRNA export and less so on AdML mRNA or snRNA export. This pattern of inhibition mimics the inhibition seen after nuclear injection of an excess of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 protein (43, 44) or the DHFR mRNA itself (3, 32). The inability of the M10 conjugate to interfere with Rev-mediated export (data not shown) or the shuttling of RanBP1 (Fig. 4) indicates that it targets a specific receptor. Thus, the M10 peptide conjugate may affect interactions such as those between hnRNP A1 protein and its cognate nuclear export receptor. The inhibition of export of certain mRNAs by M10 conjugate observed here might also explain why expression of M10 mutant Rev protein is toxic in stably transformed CMT3 cells (M.-L. Hammarskjöld, personal communication) and in *Saccharomyces cerevisiae* (C. M. Hammell and C. N. Cole, unpublished results cited in ref. 45). Because of the ability of this conjugate to inhibit mRNA export, its use as an "inactive" control for Rev NES conjugates in other studies may be problematic.

The stronger inhibitory activity of the PKI NES peptide conjugate relative to the Rev NES correlates well with the greater ability of the PKI NES to support the formation of a trimeric complex of NES peptide, exportin 1, and RanGTP *in vitro* (20). The differences between the NESs could result from inherent affinities of their "minimal" NES sequences for a receptor or from differences in the contexts in which they are presented to a receptor. Context certainly seems to be important, as a version of the PKI NES that is longer than the one used here (GSNELALKLAGLDINKTGGC; *cf.* ref. 4) is an even more effective inhibitor of RNA export (data not shown). Similarly, the addition of an aspartic acid residue to the C terminus of the Rev NES modestly increased the ability of that NES conjugate to inhibit RNA export, in keeping with the finding that this residue is important for the function of Rev protein in the expression of nonspliced HIV pre-mRNA in mammalian cells (1, 46). However, these changes in overall efficiency of inhibition do not alter the spectrum of targets affected. For example, addition of the extra aspartic acid residue did not differentially affect the ability of the REV NES conjugate to block mRNA export relative to snRNA export. Similarly, a shorter version of the PKI NES (LALKLAGLDI) inhibited the export of mRNAs and snRNAs comparably (data not shown).

The NES conjugates also affected the localization of at least one transport factor. Injection of either the PKI or the Rev NES conjugate led to nuclear accumulation of 10–20% of the normally cytoplasmic RanBP1. Because RanBP1 contains a functional NES, it has been proposed to shuttle between the nucleus and cytoplasm (38, 39); inappropriate accumulation of this protein in the nucleus might also contribute to the overall inhibition of nuclear export. In contrast, the distributions of several other transport factors such as importin α, importin β, exportin 1, Ran, and RanGAP were not significantly affected by the conjugates. The distribution of importin β, which appears to have an NES (47), is not affected by the NES conjugate, indicating that this NES may not be required for its recycling in vertebrate cells. Importin α and Ran are exported by using other export receptors (48), which might not be sensitive to the inhibitory actions of the NES conjugates.

We have shown here that mRNA export in vertebrates can be blocked by NES conjugates, indicating that it utilizes NES-containing proteins and thus resembles mRNA export in yeast. Our results do not yet resolve the question as to whether all NES-dependent export uses a single NES receptor (e.g., exportin 1) or multiple receptors; however, the selective inhibition of the export of certain RNAs by the M10 and the BSA-R conjugates is consistent with the latter possibility. In either case, the finding that mRNA export occurs in a manner similar to that of snRNAs, 5S rRNA, and HIV Rev-mediated RNA export demonstrates that general features exist and that these are likely to be mechanistically similar in yeast and vertebrates.

We thank Ariane Grandjean and Susanne Imboden for excellent technical assistance and Dr. Frank Masiarz, Chiron Corporation, for N-terminal protein sequencing of the peptide conjugates. We thank Drs. Katharine Ullman, Christian Grimm, and Jeannine Petersen for stimulating discussions. We also thank Dr. Susan Taylor (University of California, San Diego) for the long version of the PKI NES peptide and Dr. Utz Fischer for providing the BSA-R REV peptide conjugate. We thank Drs. Mary Dasso, Frank Grosveld, Dirk Görlich, and Mary Moore for generously providing us with antibodies. This work was supported by National Institutes of Health Grant GM30220 (to J.E.D.) and National Institutes of Health Grant GM33279 and American Cancer Society Grant CB199 (to D.F.). A.E.P. was supported in part by a gift from the Lucille Markey Foundation and a National Institutes of Health predoctoral training grant.

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